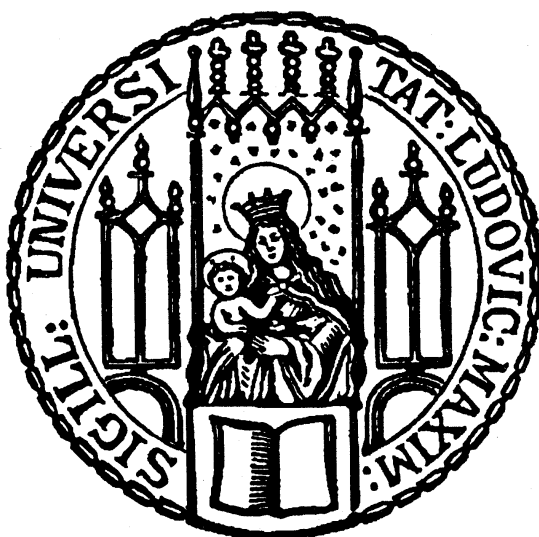


Dissertation

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Ludwigs-Maximilians-Universität München

Formulation of PEGylated and HESylated Biopharmaceuticals



von

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Erklärung

Diese Dissertation wurde im Sinne von § 7 der Promotionsordnung vom 28. November 2011 von Herrn Prof. Dr. Gerhard Winter betreut.

Eidesstattliche Versicherung

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

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.....

(Robert Liebner)

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Für meine Familie in Liebe und Dankbarkeit.

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Table of Contents

Chapter I - General Introduction 1

1. General Introduction	3
1.1. Half-life modulation of biologics	3
1.2. The bioconjugation polymer PEG and the effect of PEGylation on biologics .	6
1.3. PEGylation chemistry and PEG reagents	7
1.3.1. Random PEGylation	7
1.3.2. Site-specific PEGylation of the N-terminus	9
1.3.3. Site-specific PEGylation of thiol groups	10
1.3.4. Further strategies for PEGylation	13
1.4. Limitations of PEG and PEGylation technology	13
1.4.1. Quality of the polymer and PEGylation chemistry	13
1.4.2. Effect on activity upon conjugation	14
1.4.3. Toxicity	15
1.4.4. Immunogenicity	16
1.4.5. Effect on protein stability	17
1.4.6. Effect on viscosity	17
1.4.7. Behavior during and after lyophilization	18
1.5. Alternative strategies for half-life extension based on biodegradable polymers	19
1.6. HES and HESylation	19
1.6.1. HESylation chemistry and HES reagents	21
1.6.2. Random HESylation	22
1.6.3. Site-specific HESylation	23
1.6.4. Quality of the polymer HES	25
1.6.5. Activity	25
1.6.6. Toxicity	26
1.6.7. Effect on protein stability	26
1.6.8. Effect on viscosity	27
1.6.9. Lyophilization	27
1.7. Polysialylation	28

1.8.	Recombinant PEG mimetics	28
1.8.1.	PASylation	28
1.8.2.	XTENylation	29
1.9.	Aims of the thesis	31
1.10.	References	32
Chapter II – Half-life extension through HESylation.....		45
2.	Protein HESylation for half-life extension: Synthesis, characterization and pharmacokinetics of HESylated anakinra	47
2.1.	Introduction	49
2.2.	Experimental procedures.....	50
2.2.1.	Materials.....	50
2.2.2.	Synthesis and purification of HESylated anakinra.....	50
2.2.3.	Determination of conjugate concentration	51
2.2.4.	SEC-MALLS measurement for the determination of the molar mass and size of the conjugates	51
2.2.5.	Dynamic light scattering (DLS)	52
2.2.6.	Fourier transform infrared spectroscopy	52
2.2.7.	Surface plasmon resonance measurements for <i>in vitro</i> binding affinity	52
2.2.8.	Microscale Thermophoresis (MST) for <i>in vitro</i> binding affinity	53
2.2.9.	Microcalorimetry.....	53
2.2.10.	Thermal stability	54
2.2.11.	Pharmacokinetic study	54
2.3.	Results	55
2.3.1.	Molar mass and size	55
2.3.2.	Protein conformation.....	57
2.3.3.	Microcalorimetry and thermal stability.....	58
2.3.4.	<i>In vitro</i> binding affinity	60
2.3.5.	Pharmacokinetic profiles of native and HESylated anakinra.....	62
2.4.	Discussion	64
2.5.	Conclusions	68
2.6.	References	68

Chapter III – Highly concentrated formulations..... 75

3. Head to Head Comparison of the Formulation and Stability of Concentrated Solutions of HESylated versus PEGylated Anakinra	77
3.1. Introduction.....	79
3.2. Materials and Methods.....	80
3.2.1. Materials	80
3.2.2. Synthesis and Purification of the Protein Conjugates.....	80
3.2.2.1. PEGylation.....	80
3.2.2.2. HESylation.....	81
3.2.3. SEC–MALLS Measurement for the Determination of the Molecular Weight and Size of the Conjugates	82
3.2.4. Hydrodynamic Radius	82
3.2.5. FTIR.....	83
3.2.6. Biacore Analysis for <i>in vitro</i> Binding Affinity	83
3.2.7. Preparation of Highly Concentrated Solutions	83
3.2.8. Differential Scanning Calorimetry.....	84
3.2.9. Viscosity	84
3.2.10. Storage Stability: SEC	84
3.3. Results.....	85
3.3.1. Synthesis and Purification	85
3.3.2. Determination of the Molecular Weight and Hydro-dynamic Size.....	85
3.3.3. FTIR Analyses for Changes in Secondary Structure	87
3.3.4. <i>In Vitro</i> Binding Affinity	88
3.3.5. Properties of Highly Concentrated Solutions	90
3.3.5.1. Viscosity Measurement.....	90
3.3.5.2. Thermal Analysis	92
3.3.5.3. Thermal Stability upon Storage at 40°C	94
3.4. Discussion.....	95
3.5. Conclusion	99
3.6. References.....	100

Chapter IV – Freeze- dried formulations107

4. Freeze-drying of HESylated IFN α -2b: Effect of HESylation on storage stability and benchmarking to PEGylation	109
4.1. Introduction	111
4.2. Materials and Methods	113
4.2.1. Materials.....	113
4.2.2. Synthesis and purification of HESylated interferon α -2b	113
4.2.3. Synthesis and purification of PEGylated interferon α -2b	114
4.2.4. Synthesis and purification of HESylated rh-met-G-CSF	115
4.2.5. Pretreatment of PEGylated rh-met-G-CSF	115
4.2.6. Quality control of protein conjugates	116
4.2.6.1. UV-measurement	116
4.2.6.2. SDS-PAGE.....	116
4.2.6.3. Size-exclusion and reversed phase chromatography	116
4.2.6.4. AF4-MALLS measurement for the determination of the molar mass, size of the conjugates and conjugate stoichiometry.....	117
4.2.6.5. Dynamic light scattering (DLS)	117
4.2.6.6. Peptide mapping	117
4.2.7. Freeze-Drying Microscopy	118
4.2.8. Sample preparation for lyophilization.....	118
4.2.9. Tg' measurement.....	118
4.2.10. Freeze-drying protocol	119
4.2.11. Tg and degree of crystallization	119
4.2.12. Residual moisture	119
4.2.13. Turbidity measurement	119
4.2.14. Particle count - Light obscuration	120
4.2.15. Monomer recovery – Size exclusion chromatography.....	120
4.2.16. Conformational changes - FTIR.....	120
4.3. Results	120
4.3.1. Preparation and quality control of the polymer modified interferon samples	120
4.3.2. Lyomicroscopy.....	126

4.3.3.	Thermal analysis of the frozen formulations	127
4.3.4.	Optical evaluation of the freeze-dried samples.....	128
4.3.5.	Cake properties after lyophilization and storage	128
4.3.5.1.	Glass transition temperature and degree of crystallization.....	128
4.3.5.2.	Residual moisture	129
4.3.5.3.	Conformational stability after lyophilization and storage	130
4.3.6.	Colloidal stability after lyophilization and storage.....	131
4.3.6.1.	Turbidity and particle counts	131
4.3.6.2.	Monomer recovery and soluble aggregates	132
4.4.	Discussion.....	133
4.5.	Conclusion	136
4.6.	References.....	137
Chapter V – Highly concentrated lyophilized formulations.....		143
5.	Protein HESylation for the use of highly concentrated and freeze-dried formulations of HES-anakinra conjugates: storage stability and the benchmark to PEGylation.....	145
5.1.	Introduction.....	147
5.2.	Materials and Methods.....	148
5.2.1.	Synthesis and purification.....	148
5.2.2.	Sample preparation	148
5.2.3.	Tg' measurement	149
5.2.4.	Freeze-drying protocol.....	149
5.2.5.	Tg and degree of crystallization.....	150
5.2.6.	Residual moisture	150
5.2.7.	Turbidity measurement	150
5.2.8.	Particle count - Light obscuration.....	151
5.2.9.	Monomer recovery – Size exclusion chromatography	151
5.3.	Results.....	151
5.3.1.	Cake properties after lyophilization and storage	152
5.3.1.1.	Glass transition temperature and residual moisture.....	152
5.3.1.2.	Degree of crystallization	155
5.3.2.	Protein quality after reconstitution	156

5.3.2.1. Colloidal stability after lyophilization and storage	156
5.3.2.2. Monomer recovery and soluble aggregates.....	156
5.4. Discussion	159
5.5. Conclusion.....	161
5.6. References	162
Chapter VI – Summary and attachments.....	165
6. Summary of the thesis	167
7. Publications associated with this thesis.....	171
7.1. Research articles.....	171
7.2. Book chapter	171
8. Presentations associated with this thesis	171
8.1. Oral presentations.....	171
8.2. Poster presentations.....	172

List of Abbreviations

ABC	Accelerated blood clearance
API	Active pharmaceutical ingredient
AUC	Area under the curve
BfArM	Federal Institute for Drugs and Medical Devices
BSA	Bovine serum albumin
CA	Columinic acid
CD	Circular dichroism
CD20	B-lymphocyte antigen 20
CHO	Chinese hamster ovary
CL	Clearance
c_{\max}	Maximum concentration of a drug observed after its administration
CSE	Citrate-saline-ethylenediaminetetraacetic acid
DLS	Dynamic light scattering
DMARDs	Disease modifying antirheumatic drugs
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
<i>E. coli</i>	Escherichia coli
EPO	Erythropoietin
FcRn	Neonatal Fc receptor
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
G-CSF	Granulocyte colony-stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HES	Hydroxyethyl starch
hGH	Human growth hormone
HLE	Half-life extension
HSA	Human serum albumin
IFN	Interferon
ITC	Isothermal calorimetry
kDa	Kilodalton

LO	Light obscuration
mAb	Monoclonal antibodies
MHC	Major histocompatibility complex
M _n	Number average molar mass
MS	Molar substitution
MST	Microscale Thermophoresis
M _w	Mass average molar mass
MW	Molar mass
MWCO	molecular weight cutoff
NaCl	Sodium chloride
Neu5Ac	5-N-acetylneuraminic acid
NH ₄ OH	Ammonium hydroxide
P-20	Polysorbate 20
PAS	Peptide based on proline, alanine and serine
PD	Pharmacodynamics
PDI	Polydispersity Index
PEG	Polyethylene glycol
Ph. Eur.	Pharmacopoea Europaea
PK	Pharmacokinetic
PPI	Protein- protein- interaction
PRAC	Pharmacovigilance Risk Assessment Committee
PSA	Polysialic acid
rhIL1-ra	Recombinant human interleukin-1 receptor antagonist
RI	Refractive index
RNA	Ribonucleic acid
RP-HPLC	Reversed phase High-performance liquid chromatography
rpm	Rounds per minute
s.c.	Subcutaneous
scFv	Single chain Fv regions
SCID	Severe combined immunodeficiency disease
SE- HPLC	Size exclusion High-performance liquid chromatography
SEC-MALLS	Size exclusion chromatography - multi angle laser light scattering
SPR	Surface plasmon resonance
t _{1/2}	Half-life

T _c	Critical collapse temperature
TFF	Tangential flow filtration
T _g	Glass transition temperature
T _g '	Glass transition temperatures of the maximally freeze-concentrated matrix
T _m	Melting temperature
TNF- α	Tumor necrosis factor alpha
TRIS	Tris(hydroxymethyl)aminomethane
USP	United States Pharmacopeia
UV-VIS	Ultraviolet–visible
VEGF	Vascular endothelial growth factor

Chapter I - General Introduction

1. General Introduction

1.1. Half-life modulation of biologics

The discovery of recombinant DNA technology in the early 1980s has led to a rapidly expanding market for diagnostic and therapeutic biologics covering a broad range of human illnesses. Today, protein- and peptide-based drugs comprise over 200 approved products and hundreds of potential candidates in clinical trials. These compounds can be classified as biopharmaceuticals, which 1) mimic native proteins and operate as replacement therapies, 2) serve as antagonist therapy or 3) stimulate and mobilize malfunctioning body proteins [1]. Problems with biopharmaceuticals often involve a suboptimal physicochemical profile, typically caused by either a tendency to aggregate, limited solubility or proteolytic instability. Additionally, a molecular weight below the renal cutoff ($MW < 60$ kDa) can restrain their pharmacokinetic effectiveness, resulting in a plasma half-life of just minutes to hours. Monoclonal antibodies (mAbs) tend to avoid this issue, with a molecular weight of around 150 kDa and a naturally-mediated FcRn recycling mechanism, which together yield a plasma half-life of days to weeks [2]. Rapid elimination is thus associated with hydrodynamically smaller varieties of biopharmaceuticals such as cytokines, growth factors, peptides and protein scaffolds. The efficiency of these drugs is limited by their short circulation time, which must be overcome by frequent injections [3]. However, simultaneous circulation time enhancement and improved physicochemical properties can be obtained by covalent linkage of the active pharmaceutical ingredient (API) to biocompatible polymers. Among the first successful attempts in this regard were the experiments performed by Davies and Abuchowsky in the 1970s, who improved blood circulation of bovine liver catalase and bovine serum albumin by the chemical attachment of polyethylene glycol (PEG) [4, 5]. The first PEGylated protein was approved by the Food and Drug Administration (FDA) in the early 1990s: the PEGylated version of the adenosine deaminase (Adagen[®]) for the treatment of severe combined immunodeficiency disease (SCID), an autosomal recessive genetic disorder induced by adenosine deficiency. At least ten PEGylated biopharmaceuticals are approved today and PEGylation is considered to be the gold standard for half-life extension (HLE) [6]. **Table 1** summarizes the commercial benefit of the PEGylated products currently on the market (adapted from [7]).

Therapeutic compounds usually profit from conjugation with PEG. PEGylation reduces glomerular filtration by substantially increasing hydrodynamic size to above the renal cut-off, thereby slowing down kidney clearance. Additional benefits include protection of the drug

from interactions with catabolic and proteolytic factors and the immune system [8, 9]. Physicochemical properties are likewise improved, due to an increase in thermal stability, attenuated aggregation and enhanced solubility [10].

Most of the approved biopharmaceutical drugs are recombinant replicas of naturally occurring human proteins. Next generation biologics include an emerging class of alternative protein scaffolds like affibodies, Adnectins, anticallins or DARPins [11], which are engineered to recognize particular target structures. These small, specific binders are designed to have affinity to common targets such as TNF- α , CD20 or VEGF and are based on a robust, single-chain polypeptide framework with remarkable conformational tolerance [12-14]. As with the first generation of biologics, rapid elimination by the kidneys could prove to be the Achilles' heel of these highly specific, unique molecules. Therefore, half-life extension technologies can be expected to play an important role in market entry.

Drug	PEGylated protein	Market entry	Sales 2013 (M US\$)¹	Indication	Company
PEG–adenosine deaminase (Adagen [®])	Adenosine deaminase	1990	65	Severe combined immunodeficiency disease (SCID)	Enzon
PEG–asparaginase (Oncaspar [®])	Asparaginase	1994	55	Acute lymphoblastic leukemia	Enzon
PEG–interferon α -2b (PegIntron [®])	Interferon α -2b	2000	496	Hepatitis C	Schering-Plough
PEG-interferon α -2a (Pegasys [®])	Interferon α -2a	2002	1,416	Hepatitis C	Roche
Pegvisomant, (Somavert [®])	Growth hormone receptor antagonist	2002	217	Acromegaly	Pfizer
PEG-filgrastim (Neulasta [®])	Granulocyte colony stimulating factor	2002	4,392	Neutropenia	Amgen
Pegaptanib (Macugen TM)	PEG-anti-VEGF aptamer	2004	8	Wet age-related macular degeneration	Eyetech Pharmaceuticals/ Pfizer
PEG-epoetin- β (Mircera [®])	Erythropoietin	2007	459	Renal anemia	Roche
Certolizumab Pegol (Cimzia [®])	Fab fragment against TNF- α	2008	789	Rheumatoid arthritis and Crohn's disease	UCB
Pegloticase (Krystexxa [®])	Urate oxidase	2010	26	Chronic gout	Savient Pharmaceuticals
Peginesatide (Omontys [®])*	Erythropoiesis stimulating agent	2012	n.a.	Anemia due to chronic kidney disease	Affymax and Takeda

¹From www.evaluategroup.com

*recalled in 2013 and now withdrawn from the market

Table 1: Marketed PEGylated proteins and peptides, their year of approval, global sales in 2013 and the companies which first commercialized them (adapted from [7])

1.2. The bioconjugation polymer PEG and the effect of PEGylation on biologics

As the name suggests, polyethylene glycol is a nonionic polyether with a chemical structure of $\text{HO}(\text{CH}_2\text{CH}_2\text{O})_n\text{H}$ which can be synthesized by an anionic ring opening polymerization of ethylene oxide initiated by nucleophilic attack of a hydroxide ion on the epoxide ring [15]. However, when PEG is used for polypeptide modification, it must typically be of the heterobifunctional variety. Specifically, one of the hydroxyl end groups must be capped with a methyl group to create a monomethoxylated PEG (structure mPEG: $\text{CH}_3\text{O} - (\text{CH}_2\text{CH}_2\text{O})_n - \text{CH}_2\text{CH}_2\text{OH}$), while the second end group is modified with a functional group amenable to the conjugation step. This heterobifunctionality enables straightforward conjugation while preventing crosslinking of multiple polypeptides. In this approach, synthesis is initiated by nucleophilic attack of a methoxide ion, as opposed to hydroxide, on the epoxide ring. The final product is amphiphilic in nature. The oxygen molecules are responsible for PEG's hydrophilic character; while the hydrophobic tendency is caused by the ethylene subunits. As such, PEG is a surface active molecule, soluble both in water and in a number of organic solvents [16]. Its solubility in water over a wide range of molar masses is especially remarkable due to the fact that its two neighbors namely poly(methylene glycol) and poly(propylene glycol), are insoluble in water [17]. The apparently more polar character of PEG is derived from a strong tendency of the oxygen atoms to form hydrogen bonds between 2-3 water molecules; this results in extraordinary hydration of the polymer with high conformational flexibility and chain mobility [17]. The exact water-binding capacity ranges from 2-3 water molecules per subunit up to 16 molecules [18, 19] depending on the method used for quantification [20, 21]. In general, PEG is considered to be non-toxic, non-immunogenic and biocompatible and is therefore approved by the FDA for parenteral usage [22]. Coupling the API of interest to PEG will in most cases drastically improve the physicochemical properties of the conjugate. For instance, hydrophobic drugs become soluble in an aqueous environment after PEGylation. One of the most oft-cited examples of this phenomenon is interferon β -1b [8]. Native IFN β -1b is indicated for the treatment of multiple sclerosis and approved as Betaferon[®] (Bayer HealthCare) or Extavia[®] (Novartis). However, this formulation requires the addition of human serum albumin to stabilize the protein and preserve solubility after reconstitution. Basu *et al.* reported that unmodified IFN β -1b began to precipitate as insoluble aggregates within 7 days at neutral pH in the absence of a detergent. In contrast, covalent coupling of a 40 kDa PEG was able to maintain solubility during that time [8]. The modified protein also profited from

an improved pharmacokinetic profile, a lower tendency toward aggregation and reduced immunogenicity [8].

1.3. PEGylation chemistry and PEG reagents

1.3.1. Random PEGylation

For conjugation to therapeutic proteins and peptides, pharmaceutical grade PEG reagents are commercially available in linear or branched architectures and with a variety of different end group linker moieties for subsequent coupling. These linkers can either react directly or after an activation step with particular functional groups on the surface of the protein, in both cases forming a covalent bond. When linking to proteins, available conjugation targets for PEG include amino acids like lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine and tyrosine, as well as the N-terminal amino group and the C-terminal carboxylic acid [19]. For random PEGylation, lysine, with its primary amine side group, is an attractive target due to the fact that it can represent up to 10% of the primary sequence in many proteins. This ϵ -amino group represents a nucleophilic target for a number of electrophilic functional groups. As a result, it is unsurprising that “first-generation PEG chemistry” included PEG polymers modified with (1) dichlorotriazine, (2) tresylate, (3a) succinimidyl carbonate, (3b) benzotriazole carbonate, (3c) p-nitrophenyl carbonate, (3d) trichlorophenyl carbonate, (4) carbonylimidazole and (5) succinimidyl succinate end groups, all of which attack the abundant ϵ -amino groups found in proteins (**Figure 1**).

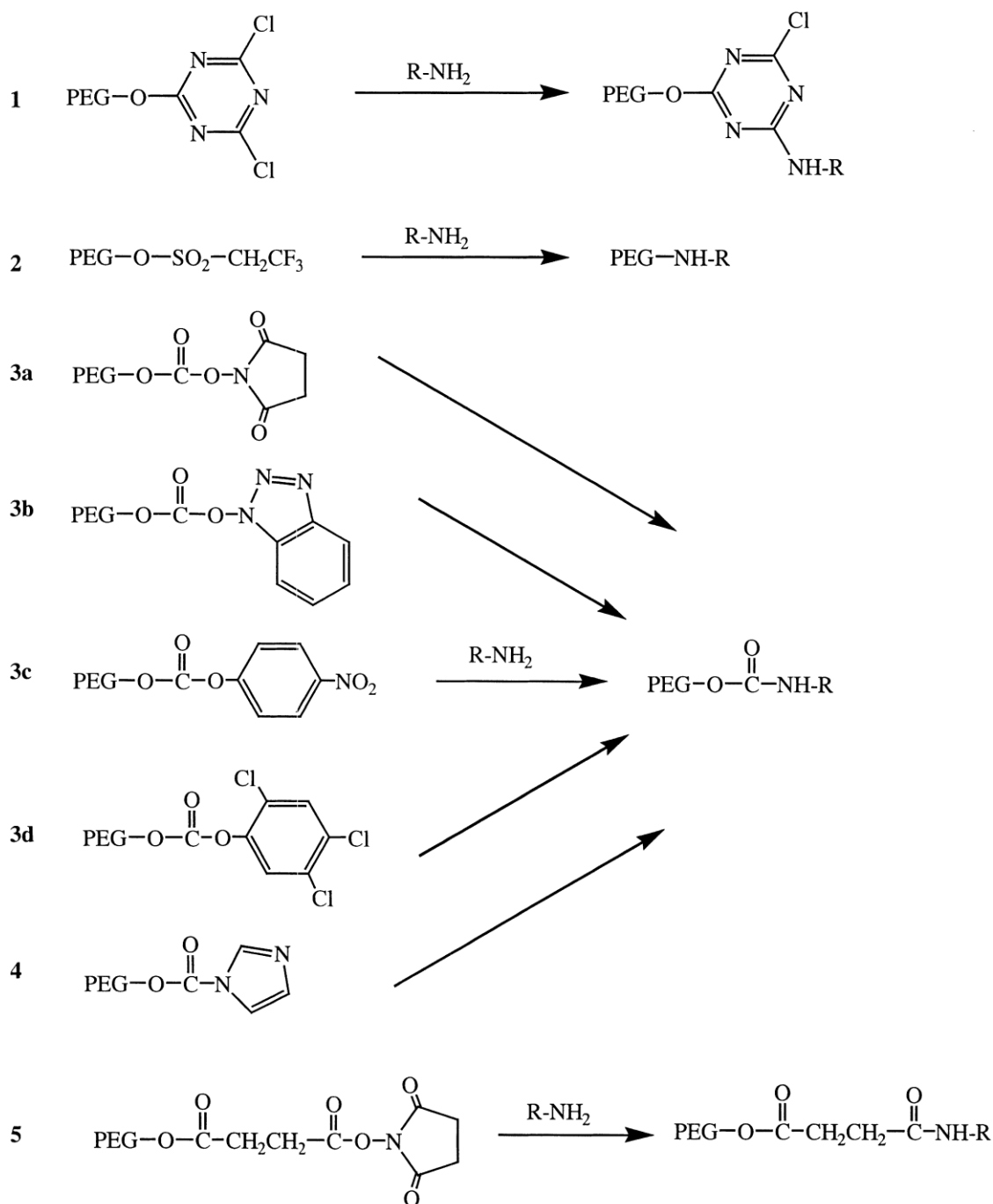


Figure 1: Activated PEG-derivatives for the chemical coupling to ϵ -amino groups
(adapted from [19])

Such reactions are rapid and straightforward to optimize and scale, but are dominated by a lack of selectivity, resulting in a number of positional isoforms and differences in the total number of coupled PEG chains per protein [19]. The reactivity of the functional group and the protein to PEG ratio control the prevalence of side reactions with other nucleophiles on the protein surface, namely the N-terminal amino group, the imidazole nitrogens of histidine residues and the side chains of serine, threonine, tyrosine and cysteine residues [23]. The exist-

ence of a number of positional isoforms led to concerns about the reproducibility of drug batches and may have contributed to higher antigenicity of the modified drug and poor clinical outcomes [3]. Furthermore, unstable linkages between PEG and the protein were sometimes used, which triggered degradation of the PEGylated drug during manufacturing and storage [24]. An additional problem was caused by the presence of diols, representing up to 15% by mass in batches of mPEG, which resulted in API crosslinking and the formation of aggregates [3, 19]. However, several PEG conjugates which emerged from “first-generation PEG chemistry,” such as Adagen[®] and Oncospar[®], did in fact receive regulatory approval. The historical evolution of improvements to PEGylation chemistry is presented in **Table 2**.

Decade	PEG reagents	General observations	Applications
1970 – 1980	PEG-chloro triazine [5] PEG-succinimidyl succinate PEG-tresyl	Immunogenic or toxic starting material, highly polydisperse PEG, lack of selectivity	Research studies, enzyme modification for biocatalysis
1980 – 1990	PEG-aldehyde [25] PEG-succinimidyl carbonate PEG-pNO ₂ phenyl carbonate PEG-AA-NHS [26] PEG-carbonylimidazole, etc.	Site-specific conjugation, less polydisperse PEG, absence of diols	Enzyme replacement therapy
1990 – 2000 [27]	Branched PEG PEG-NHS PEG-maleimide PEG-OPSS	Improved selectivity, marketing of PEGylated drugs	Cytokines, hormones, anticancer drug targeting
2000 - 2014	Enzymatic coupling [28] Disulfide coupling [29] Releasable PEGs [30] Forked PEGs Star PEGs Monodisperse PEGs	Detailed chemical and biological characterization of conjugates, combination of genetic engineering and PEGylation in the design and discovery of new drugs, more stringent regulatory requirements	Non-protein drug PEGylation, oligonucleotide PEGylation, cell PEGylation
AA = amino acids; NHS = N-hydroxysuccinimide; OPSS = ortho-pyridyldisulfide			

Table 2: History of PEGylation (adapted from [31])

1.3.2. Site-specific PEGylation of the N-terminus

PEG chemistry from the so-called “second generation” was developed to eliminate first-generation pitfalls by reducing polydispersity (also for high molecular weight PEGs) and diol content. Improvements to the stability of the linkers were also introduced by creating new functional moieties that enabled a more tunable conjugation process. The use of propionalde-

hyde linkers in combination with a reducing agent such as sodium cyanoborohydride facilitated the linkage to the α -amino acid of a protein's N-terminus under mildly acidic conditions (e.g. pH 5.0). This reaction takes place preferentially at the N-terminal amino acid due to the difference in the pKa value of ϵ -amino groups of lysine residues, which have a pKa of 10.1, and the pKa value of the N-terminal amino group, which has a pKa of 7.8 [25, 32, 33]. In the first step of this reaction, a labile Schiff's base is formed, which is subsequently reduced by sodium cyanoborohydride to a stable secondary amine (**Figure 2**) [34].

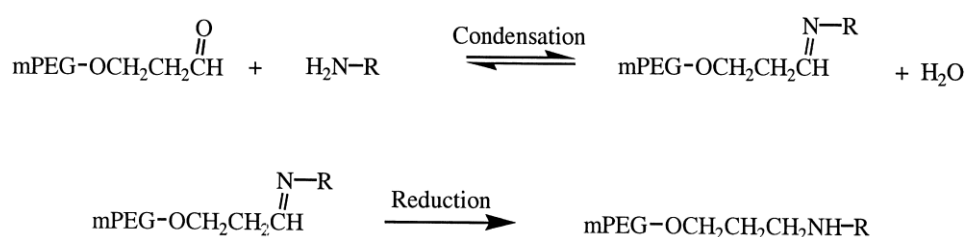


Figure 2: Reductive amination using PEG-propionaldehyde (adapted from [19])

1.3.3. Site-specific PEGylation of thiol groups

The thiol group of an unpaired cysteine residue is rarely found in native proteins, as it is usually involved in a disulfide bridge with another cysteine residue [6, 35]. With genetic engineering, however, it is possible to produce recombinant proteins containing an unpaired cysteine residue. Therefore, thiol PEGylation is not limited to proteins with a naturally occurring unpaired and unprotonated thiol group [6]. However, incorrect coupling or disulfide scrambling, which can happen during process steps like purification, pose challenging obstacles to proper expression of these proteins. If protein dimerization via a disulfide bond takes place, the yield of coupling efficiency can be quite low under this approach [6]. If this is not a significant issue, however, selective linkage of PEG to the thiol groups of unpaired cysteine residues can be achieved by use of a number of reactive groups such as maleimide, vinylsulfone or iodoacetamide, which all form stable thioethers (**Figure 3**). The most common form of thiol coupling is the use of PEG derivatives carrying a maleimide end group, which forms a thioether bond with unpaired sulfhydryl groups under neutral conditions through a Michael's addition [6].

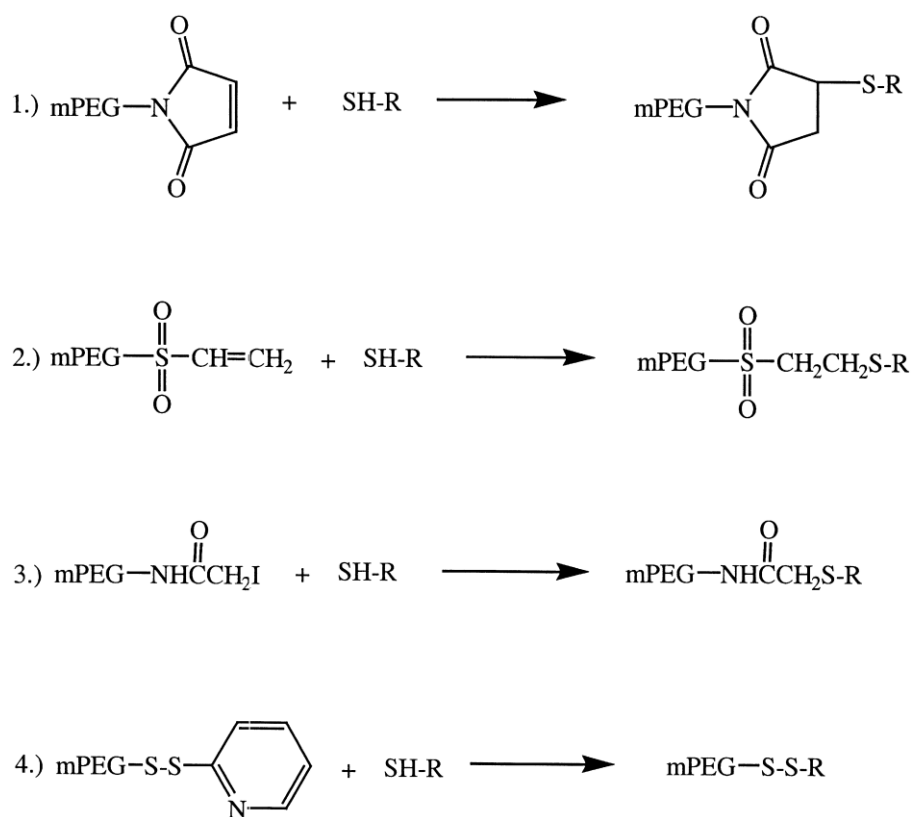


Figure 3: Thiol reactive PEGs. (1) PEG maleimide, (2) PEG vinylsulfone, (3) PEG iodoacetamid and (4) PEG orthopyridyl disulfide

Table 3 illustrates the different PEG agents used for approved PEG-drug conjugates, including MW, linker structure and type of modification (adapted from [36]).

Trade name	Drug	PEG agent (MW and linker structure)	Type of modification
Adagen®	PEG-adenosine deaminase	Linear 5 kDa / NHS-ester	HyperPEGylation – random on predominantly ϵ -amino groups
Oncaspar®	PEG-asparaginase	Linear 5 kDa / NHS-carbonate	HyperPEGylation – random on predominantly ϵ -amino groups
Neulasta®	PEG-G-CSF	Linear 20 kDa / PEG-aldehyde and reducing agent	MonoPEGylation – specific N-terminal α -amino group
PegIntron®	PEG-interferon α -2b	Linear 12 kDa / PEG-p-nitrophenyl carbonate	MonoPEGylation – random on predominantly ϵ -amino groups of lysine residues
Pegasys®	PEG-interferon α -2a	Branched 40 kDa / PEG-NHS-ester	MonoPEGylation – random on predominantly ϵ -amino groups of lysine residues
Somavert®	PEG-human growth hormone receptor antagonist	Linear 5 kDa / NHS-ester	HyperPEGylation – random on predominantly ϵ -amino groups of lysine residues
Mircera®	PEG-epoetin- β	Linear 30 kDa / PEG-aldehyde and reducing agent	MonoPEGylation – random on predominantly ϵ -amino groups of lysine residues
Cimzia®	PEG-anti-TNF- α Fab'	Branched 40 kDa / PEG-maleimide	MonoPEGylation – specific on a thiol group of unpaired cysteine residue
Krystexxa®	PEG-uricase	Linear 10 kDa / PEG-p-nitrophenyl carbonate	HyperPEGylation – random on predominantly ϵ -amino groups of lysine residues
Omontys®	PEG-hematide	Branched 40 kDa / PEG-aldehyde and reducing agent	MonoPEGylation – specific on a secondary amine group between the dimeric peptide

Table 3: PEGylation chemistry used in approved PEG-drugs

1.3.4. Further strategies for PEGylation

A number of excellent articles and reviews have described additional novel – and sometimes highly sophisticated – pathways to PEGylation [27, 37-40]. For instance, a reducible linkage can be facilitated by the formation of a disulfide bridge using PEG-orthopyridyl disulfide [19]. A variety of other linker structures and approaches have been reported to create releasable and noncovalent PEG conjugates [6]. A more exotic approach to PEGylation utilizes an enzymatic pathway, which involves the addition of transglutaminase (TGase), discovered primarily by Sato *et al.* [41]. The reaction takes place between a PEG derivative carrying a primary amino end group and the γ -carboxamide group of glutamine residues, yielding a highly selective conjugation [37, 42].

1.4. Limitations of PEG and PEGylation technology

1.4.1. Quality of the polymer and PEGylation chemistry

Polyethylene glycol is a synthetic polymer and may therefore be characterized by a certain polydispersity. Low molecular weight oligomers (3-5 kDa) have a polydispersity value (M_w/M_n) of less than 1.01; this value can increase up to 1.2 for higher molecular weight PEGs (≥ 20 kDa) [27, 43]. A broader distribution in molar mass of the PEG starting material will be directly transformed into a broader polydispersity of the PEGylated protein, which can lead to batch-to-batch variations. Consequences include changes in pharmacokinetics between batches and hampered analysis and characterization [44].

For the conjugation process, only activated PEG derivatives can be used, which may contain certain amounts of impurities that influence conjugate synthesis and stability after coupling [45]. For protein conjugation, commercially available monomethoxy PEG (mPEG) reagents can contain considerable amounts of diol PEG, up to 15% by mass, due to the presence of trace amounts of water during polymerization [19]. Another common type of contaminant in PEG polymers is peroxides. The initial amount of peroxides present at the time of manufacture can increase upon storage due to the presence of oxygen, light exposure or metal-induced auto-oxidation [46]. This can cause a loss of protein stability or activity upon PEG coupling [47]. Auto-oxidation leads to formation of hydroperoxides as well as peroxide free radicals, which promote PEG chain scission and increased polydispersity [48, 49]. This undesirable reaction is not unique to PEG and can also occur for polysorbate-based surfactants, poloxamers and other substances which contain a number of ethylene oxide units [48]. Optimized

storage conditions, such as storage away from light, under an inert atmosphere (argon or nitrogen), in the presence of antioxidants such as 2-tert-butyl-4-methoxyphenol and at temperatures below -15°C can attenuate the accumulation of peroxides [48]. Another chemical impurity was reported by Zhang *et al.*, who described the presence of a monomethoxy polyethylene glycol (mPEG)-acetaldehyde impurity in batches of mPEG-aldehyde. Storage of a PEGylated protein derived from this raw material for 12 months at 2-8°C initiated a slow hydrolysis of the acetal bond, which resulted in dePEGylation of the protein [50]. Finally, contaminants can impact the reactivity of the functionalized PEG polymer, influencing the degree of PEGylation during the conjugation process and leading to batch-to-batch variations; as such, reactivity of the PEG derivative must be evaluated before each conjugation step [49].

Today, a number of different coupling strategies are available to attach functionalized PEG polymers to nucleophilic targets such as amino or thiol groups on the protein surface. Thiol coupling performed by maleimide chemistry is not stable under alkaline conditions and can undergo ring opening leading to release of the protein. PEG reagents carrying an iodo-containing active group can generate iodine during the conjugation, which can interact with tyrosine residues. Succinimidyl esters do not couple selectively to amino groups and can also react with tyrosine and cysteine residues, creating unstable linkages that can slowly hydrolyze during storage [51]. The formation of multi-PEGylated species can drastically reduce the yield of the desired mono-PEGylated drug [52]. Additionally, positional isomers of a PEGylated protein cause heterogeneity in the final product [49]. In conclusion, comprehensive quality control of the raw material, validation of the conjugation process and a well-designed battery of physicochemical characterization methods [50] are required to provide the consistent quality and reproducibility of the PEGylated drug needed to obtain approval by regulatory authorities and ensure patient safety [53].

1.4.2. Effect on activity upon conjugation

PEGylation tends to decrease the *in vitro* and *in vivo* activity of the protein, sometimes drastically. This diminished activity is not surprising; PEG has a high shielding effect, protecting proteins from unwanted interactions but also impeding target or receptor recognition. A detailed discussion is given by Kubetzko *et al.*, who pointed out that the decline in conjugate activity is due to decreased association of the conjugate with the binding partner. The dissociation constant, in contrast, is in general unaffected [54]. In most cases, activity of a protein is related to a short sequence in the primary structure. Therefore, the conjugation site plays a major role. Basu and coworkers utilized a library of different versions of PEGylated IFN β -1b

to describe how the conjugation site (random conjugation on lysine residues, N-terminal coupling or the linkage to free thiol groups) influences the conjugate's antiviral activity [8]. Pegasys[®], an approved PEGylated version of IFN α -2a made by Roche, has a very low residual activity – as low as 7% compared to the unmodified protein. However, the conjugate exhibits a 70-fold increase in serum half-life and a 50-fold increase in the mean plasma residence time in mice, outweighing the reduction in activity [55].

Size, shape and length of the polymer chains can also influence circulation time, absorption rate and biological activity. In general, an increase in chain length prolongs circulation time, but reduces residual *in vitro* activity. Branched PEGs, or a molecule comprising two chains of identical length, prolong circulation more than linear derivatives of the same nominal molecular weight, but will also reduce the residual *in vitro* activity to a greater extent than a linear chain of the same molecular weight [56].

1.4.3. Toxicity

One of the main limitations of PEG is its non-biodegradability. In general, successful prolongation of the circulation time and eventual renal filtration requires PEG of a certain molar mass, usually less than 30-40 kDa [7]. The elimination route for PEG can differ from case to case, mainly driven by the fate of the protein or peptide portion of the conjugate. For example, when a conjugate is taken up by a cell via receptor-mediated endocytosis, PEG will also be absorbed. In this case, PEG can induce vacuolization of the cell, which is not observed for administration of PEG or unmodified protein alone. This phenomenon has been reported for a number of cell types from different tissues and organ types, including the kidneys, liver, spleen and bone marrow [57, 58]. Essentially, for so long as treatment with the PEGylated drug continues, the protein will be present in cellular vacuoles, which can be verified by immunostaining [58]. If intracellular degradation of the protein portion of the conjugate takes place, the vacuoles generated will contain PEG alone, which lysosomal proteases are typically unable to degrade [59]. In theory, degradation of PEG requires an etherase, which cleaves ether linkages, but these enzymes are not commonly found in mammalian cells [58]. The degree of vacuolization and size of vacuoles formed are highly dependent on both the dosing interval and the total amount of the modified drug. Bendele and colleagues showed clear dosage dependencies for vacuolization when rats were treated with anywhere from 4 to 40 mg PEGylated API per kg body weight. The highest dosage led to the largest vacuoles in kidney tissue. After cessation of drug administration, vacuoles were not completely eliminated from the cells, even after a period of 3 months [58]. Smaller vacuoles could regress, but additional

studies by Webster *et al.* showed that larger vacuoles can persist for at least 2 months [59]. In the worst-case scenario, consequences can include a failed restoration of cells to status quo, which can then be followed by cell death [60]. During drug development, toxicity studies are routinely employed, but must be evaluated more critically when selecting dosing regimens, especially when the treatment requires chronic administration of high doses of a PEGylated drug.

1.4.4. Immunogenicity

Both PEG and PEGylated systems are widely used in pharmaceutical research, clinical applications, food additives and cosmetic products since PEG is in general non-immunogenic, biocompatible and non-toxic. However, the common use of PEG entails continuous exposure to the polymer. Even as early as 1984, Richter and Åkerblom published the existence of anti-PEG-antibodies, which were found in 0.2% of healthy test donors. By the early 2000s, this number had increased to 25%, probably due to both continuous exposure to PEG and the development of higher-sensitivity methods to detect anti-PEG antibodies [61]. In the field of PEG-coated drug-delivery systems as well as PEGylated proteins and peptides, anti-PEG-antibodies have already been described [37, 62, 63]. In these applications, the linker between polymer and conjugation site plays a remarkable role. For PEG derivatives carrying an aromatic linker or where the link is quite close to heterocyclic groups in the protein, immunogenicity of the conjugate can significantly increase [63]. Especially for PEGylated liposomes and particles, accelerated blood clearance (ABC) can occur upon second administration. This so-called ABC phenomenon includes the production of anti-PEG antibodies with an IgM subtype, which fosters rapid elimination of the PEG conjugate from the body. For approved PEGylated proteins such as asparaginase, uricase and certolizumab pegol, induction of anti-PEG antibodies and concomitant accelerated elimination has already been reported; the net effect is to increase the number of non-responders in the patient population [64-66]. Armstrong *et al.* found a clear relationship between pre-existing antibodies to PEG and diminished clinical response and suggested routine screening to monitor clearance rate and response, variables which can inform the decision to adjust dosing or administer alternative, non-PEGylated therapies [67].

1.4.5. Effect on protein stability

The physical stability of a protein is associated with two main thermodynamic aspects: colloidal and conformational stability [68]. Conformational, or thermodynamic, stability is correlated to the melting temperature (T_m) in solution, or the point at which the protein starts to unfold into a nonnative state [69, 70]. PEGylation has, in most cases, been widely reported to increase the thermodynamic stability, resulting in higher T_m values. For example, recombinant human endostatin showed an increase in melting temperature of 15°C, whereas PEGylated α -chymotrypsin was reported to have a 6°C higher T_m compared to the unmodified protein [71, 72]. In contrast, Gonnelli *et al.* found a decrease in melting temperature for PEGylated azurin; Plesner and colleagues observed a similar effect with calorimetric studies on PEGylated bovine serum albumin (BSA) over a series of different PEG chain lengths [73, 74].

PEG is of high osmotic activity [75], exhibits amphiphilic behavior and can bind to hydrophobic patches or aromatic clusters [76, 77]. In principle, specific adsorption of PEG on the surface of the protein can induce partial dehydration [73]. Furthermore, PEG can act as a precipitant due to unfavorable preferential exclusion of PEG at higher temperatures [78].

PEGylation has been widely reported to improve colloidal stability by physically separating monomers from one another, leading to reduced protein-protein-interaction and therefore, reduced aggregation [8, 79, 80]. Contrary reports given by Veronese *et al.* reported a higher aggregation tendency for PEGylated G-CSF (linked by conjugation to a buried thiol group), caused predominantly by a subtle conformational change in the protein that exposes residues with a more hydrophobic character [81]. In the end, PEGylation cannot be assumed to improve conformational and colloidal stability in general, but must be investigated on a case-by-case basis.

1.4.6. Effect on viscosity

PEG and PEGylated biopharmaceuticals exhibit nonlinear increases in viscosity with increasing concentration in solution [82, 83]. Such altered physicochemical properties are primarily driven by the architecture of the chosen PEG polymer. At higher concentrations, linear PEG polymers are especially prone to chain entanglement, which increases the viscosity of the solution. In the case of PEGylated canine hemoglobin, the viscosity increase was dependent on the number of coupled PEG molecules, whereas the viscosity of the unmodified counterpart was nearly constant over the range of measured concentrations [83]. This viscosity effect is driven by both the length of the PEG chain and its branching factor. In general, highly

branched polymers have a lower intrinsic viscosity compared to linear versions with comparable molecular weight [84]. The main cause is rooted in the different topology of linear vs. branched polymers. A highly branched polymer architecture allows the molecule to act more like a hard sphere, which is less prone to chain entanglement when compared to a linear, more flexible polymer chain.

1.4.7. Behavior during and after lyophilization

One potential drawback to use of PEG is the fact that PEG, when used as a bulking agent or when chemically grafted to a protein, tends to phase-separate during freeze-drying – an initial step toward crystallization [85, 86]. As a consequence, PEG conjugates experience a stronger tendency toward protein degradation if nascent crystallization is not suppressed by amorphous lyoprotectants and bulking agents [86, 87]. During and immediately after the lyophilization process, PEG crystallization is not immediately ruinous but will increase during storage, especially at elevated temperatures [88]. The route most commonly used to overcome crystallization is the addition of disaccharides like sucrose, which are frequently used to stabilize proteins during freeze-drying and subsequent storage in the dried state by forming hydrogen bonds that inhibit unfolding [89]. These sugars tend to remain amorphous during dehydration and can also decrease crystallization [90]. For a freeze-dried formulation of a PEGylated protein, high sucrose-to-PEG weight ratios are required (≥ 5 [88]) to suppress PEG-induced crystallization [85, 86, 91].

1.5. Alternative strategies for half-life extension based on biodegradable polymers

Half-life extension is becoming an essential component of the industrial development of small therapeutic peptides and proteins such as hormones, growth factors, cytokines, coagulation factors and enzymes [92]. PEGylation technology is by far the gold-standard for half-life extension but suffers from a number of shortcomings. The last decade has seen rapid growth in novel, alternative half-life extension technologies, including the use of other hydrophilic polymers, the development of recombinant PEG-mimicking polypeptide chains and the evolution of albumin-binding molecules. Additionally, genetic engineering of the Fc region has been used to alter the half-life of IgG molecules, opening new possibilities for the expansion of next-generation antibody-based drugs. An entire book has been written on the topic, with excellent reviews and case studies by contributors from industry and academia [92]. All available methods can be divided into two main strategies. Strategy 1: Reducing renal filtration by increasing the hydrodynamic size of the protein, which can be achieved by chemical linkage to a polymer or fusion with large recombinant polypeptides. Strategy 2: The use of methods which increase the size of the molecule and keep the drug in circulation by using the natural recycling mechanism mediated by the Fc neonatal receptor. Therefore, the molecule of interest has to be chemically linked or fused to either albumin or the Fc part of an IgG antibody [93, 94]. The following discussion is focused on techniques wherein biodegradable polymers or large polypeptides are fused to the molecule of interest.

1.6. HES and HESylation

HES is the semi-synthetic and water-soluble version of poorly soluble waxy maize starch fragments, which can be synthesized by ethylene oxide-mediated hydroxyethylation of starch under alkaline conditions (**Figure 4**). The polysaccharide is constructed of amylose (linear glucose polymer based on α -1,4-glycosidic bonds) and amylopectin (linear glucose polymer based on α -1,4-glycosidic bonds with branching points based on α -1,6-glycosidic bonds).

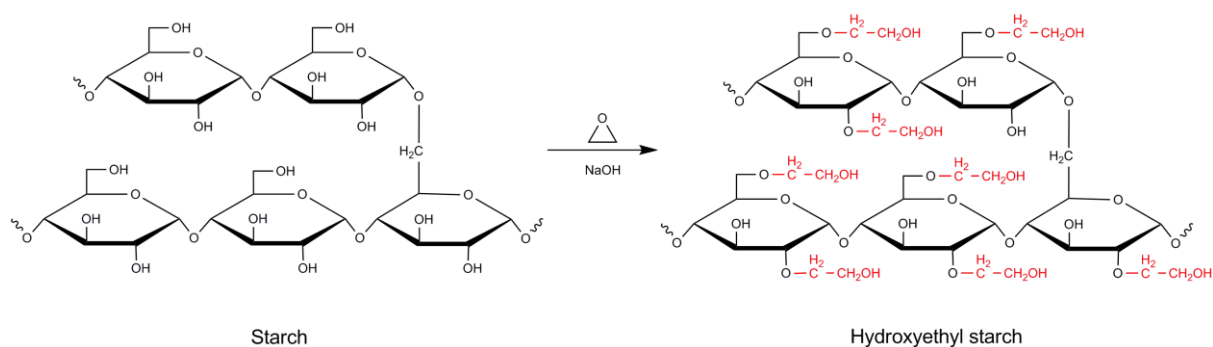


Figure 4: Hydroxyethylation of starch

The naturally occurring starch molecule exhibits a short serum half-life due to fast enzymatic digestion by serum amylase. Hydroxyethylation improves the solubility of starch in water, decreases the viscosity of starch solutions and notably lowers its biodegradability. The modification predominantly occurs at position C2, followed by C6 and C3 on the starch molecule. Hydroxyethylation at position C2 significantly hinders the degrading enzyme (α -amylase) from reaching its cleavage site and increases the circulation time. Factors like molar mass and the average number of hydroxyethyl groups per glucose subunit (C2/C6 ratio) can be a tunable tool to extend the half-life of the HES conjugates from minutes up to hours [95, 96].

The HES production process is divided into three steps (**Fig. 5**). First, amylopectin-rich starch is cleaved using acid or enzymatic hydrolysis to adjust the molecular weight. Next, the resulting starch fragments are hydroxyethylated using ethylene oxide under alkaline conditions. The degree of hydroxyethylation is controlled primarily by the reaction time. Purification and/or fractionation as a final polishing step are then applied to adjust the polydispersity of the resulting HES. A detailed description is given in the Patent EP0402724 A1 [97].

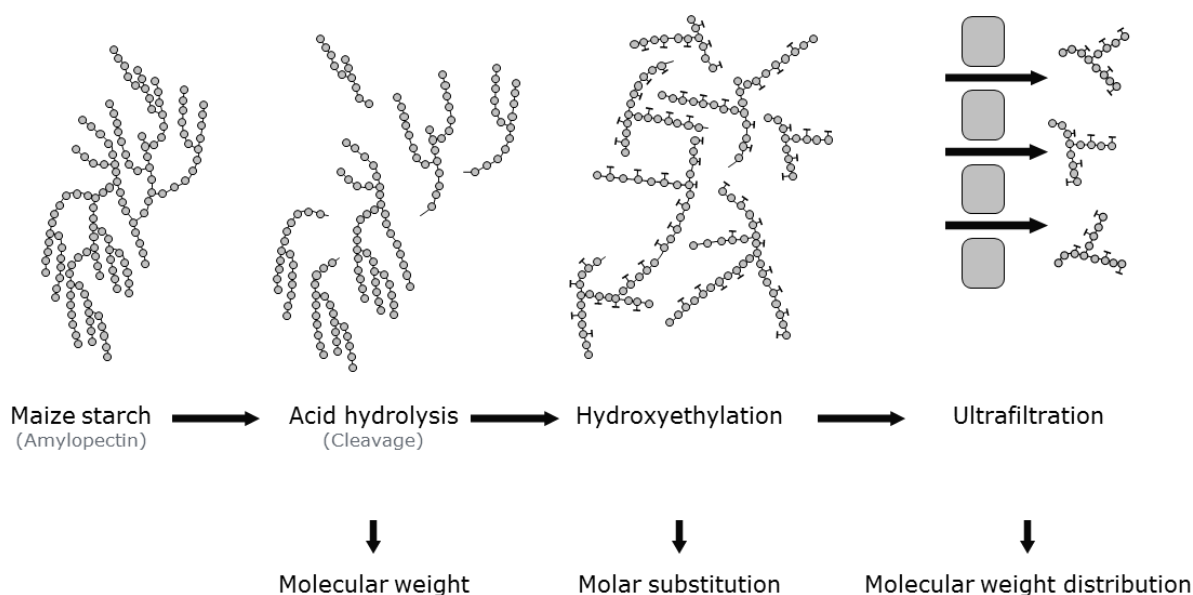


Figure 5: Preparation of hydroxyethyl starch (adapted from [98])

Hydroxyethylstarch is widely used as a plasma volume expander (PVE) due to its high biocompatibility and biodegradability, and can be administered in doses of up to 200 g/day [96]. The structural similarity to glycogen (the human glucose storage moiety) is thought to be the reason for its low immunogenicity and the correspondingly low incidence of HES hypersensitivity [99]. Additionally, and in contrast to other PVEs such as dextran or albumin, HES displays a lack of bacterial/viral contamination hazards. Undesirable drug interactions, such as the interaction of ACE inhibitors with albumin, are also absent [99].

In conclusion, HES is characterized by a combination of desirable properties like excellent biocompatibility, tunable biodegradability and high tolerable doses. As a result, HESylation for half-life extension represents a promising alternative to PEGylation technology. And in fact, global pharmaceutical companies such as Octapharma, Boehringer Ingelheim, Bayer HealthCare and Sandoz have already begun using HESylation[®] technology for the development of novel drug candidates that require extended half-life [100-103].

1.6.1. HESylation chemistry and HES reagents

HESylation, as the name suggests, involves the covalent coupling of HES to the molecule of interest. HESylation technology was first demonstrated with a HES-albumin conjugate in the 1970s by Richter and de Belder [98]. They used those conjugates to immunize rabbits and obtain antisera for HES diagnostic purposes; HES had at that point already been in clinical use for several years as a plasma volume expander [104].

1.6.2. Random HESylation

Early results for HESylated proteins such as hemoglobin were obtained by a random conjugation step involving cyanogen bromide activation of HES or a periodate oxidation to obtain an amination of the aldehyde groups for novel blood substitutes [104-106]. These studies were then supported by clinical results [107-109]. However, these HES conjugates faced some manufacturing limitations and suffered from toxicity problems. As with PEGylation, random HES linkage resulted in conjugation to a number of lysine residues with poorly controllable stoichiometry. Additionally, the multivalent HES molecules (with numerous BrCN-activated or aldehyde sites) in combination with poorly defined multivalent reaction sites often resulted in polymerization of the proteinaceous reaction partner. Tolerance of such conjugates was quite low in animal experiments due to the high fraction of covalently linked high molecular weight aggregates. Factors like stoichiometry and conjugate size were also not well-controlled under the chosen conditions [98]. At that point, the main focus shifted to use of the monovalent aldehyde functionality of the terminal glucose unit, which is not involved in glycosidic bond formation and is thus available for further derivatization steps. The reaction of hypoiodide under mild alkaline conditions resulted in selective and quantitative conversion of the aldehyde into an aldonic acid [98]. With this single carboxyl end group it was possible to HESylate by an EDC-mediated addition to protein amino groups or by forming a reactive ester using disuccinimidyl carbonate. Although reactive NHS esters are highly sensitive to hydrolysis, the HESylation achieved was sufficient to obtain high yields of HES-albumin conjugates and HES-nucleic acid molecules in aqueous solution [110, 111]. However, the high reactivity of the esters triggered the formation of unwanted linkages with thiol and protein hydroxyl groups. These drawbacks led to research activities on the implementation of specific linker structures, including use of aliphatic diamines or hexamethyldiamine to obtain an amino-HES structure. This structure was then used either as is or as an intermediate for reaction with another bifunctional linker, resulting in reactive components for thiol modifications or aldehyde- or amine-reactive functionalized groups, for example [112]. **Figure 4** illustrates a number of different coupling possibilities.

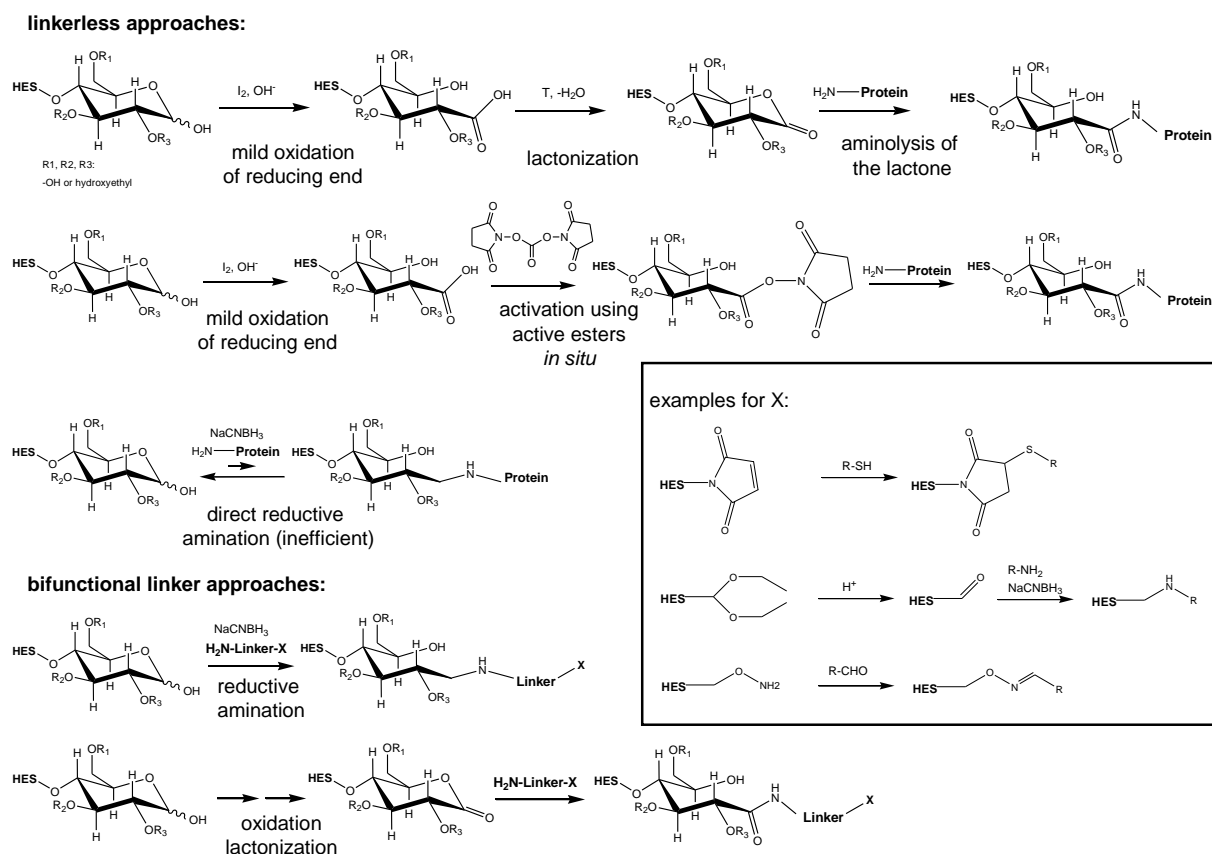


Figure 4: Chemistry of regioselective modification of the reducing end group
(adapted from [98])

1.6.3. Site-specific HESylation

HESylation[®] technology, similar to PEGylation technology, at one point faced the problem of ensuring site-directed polymer coupling with well-defined stoichiometry: characteristics which are considered advantageous from a regulatory point of view. As mentioned above, stable and ready-to-use forms with a variety of linker structures became available to fulfill such requirements [113]. Site-directed HESylation was applied to a number of low molecular weight substances like amphotericin B, peptides and proteins such as an erythropoietin mimetic peptide, erythropoietin, interferon α -2b and anakinra [98, 114, 115].

For instance, erythropoietin mimetic peptide was coupled via its single thiol group to an activated HES polymer carrying a number of maleimide groups which could link up to five molecules of the peptide while retaining their functionality. Additionally, the HESylated derivative showed excellent efficiency, better than the peptide alone and comparable to that of erythropoietin (EPO) and Aranesp[®] (Darbepoietin alpha) [116]. In the case of EPO, HESylation was

performed either at the N-terminal amino group or the glycosylation site of the protein [98]. The latter coupling was achieved by oxidation of the glycan structure using galactose oxidase to obtain an aldehyde functionality, which was used for reactive coupling to aminated HES molecules. *In vivo* studies in dogs performed for such conjugates showed that half-life can be tuned by varying the molar mass (MW) and molar substitution (MS). The HES conjugate with high MW and high MS led to threefold longer half-life compared to that of the commercially available glycosylated form of EPO, Aranesp[®]. Additionally, a PD study performed in mice showed a fourfold increase in hematocrit using HES-EPO over and above that of the unmodified counterpart and a 1.5-fold increase compared to Aranesp[®] [98]. Commercially available PEG-EPO (Mircera[®]) served as a benchmark and showed comparable *in vitro* and *in vivo* bioactivity profiles.

HESylation of the N-terminal amino group of interferon α -2b performed by regioselective conjugation with an aldehyde-containing HES derivative under acidic conditions yielded a 1:1 coupling stoichiometry > 80%. *In vivo* experiments performed in rabbits compared HES-IFN α to its PEGylated counterpart, resulting in comparable pharmacokinetics [98]. Additionally, conjugation at the N-terminus was performed in order to obtain improvements in product homogeneity relative to approved PEGylated versions of interferons such as PegIntron[®] and Pegasys[®], which both yield a number of positional isoforms upon conjugation. Both PEG-drugs are derived from a more random mono-PEGylation using NHS-activated PEG derivatives, which are known for their limited selectivity for amino groups [98].

In conclusion, several chemical options have been successfully developed for covalent coupling of HES to molecules of interest. However, chemical modifications can have some detrimental effects on very sensitive proteins. Under those circumstances, biocatalysis can be applied as a gentler alternative to strictly chemical modification strategies. Additionally, enzymes are known for their high specificity and selectivity, which can significantly increase the yield of site-specific conjugation of HES, especially for candidates which involve very complex structures. Following previous reports of enzymatically-mediated PEGylation [41, 42], a feasibility study was performed for enzymatic catalysis of HES conjugation using transglutaminase [117]. In particular, recombinant, microbially sourced transglutaminase (rMTG) catalyzes the addition of a primary amine to an acyl residue. Glutamine residues carry a gamma-carboxamide group and can act as an acyl donor. HES is then modified by esterification using N-carbobenzyloxy glutaminyl glycin and hexamethylene diamine to obtain an amino-HES, which acts as an amino donor substrate for the conjugation step. In this case, the

amino-HES derivative was used for HESylating dimethylcasein and monodansyl cadaverine [117].

1.6.4. Quality of the polymer HES

Use of HES for HESylation requires that two main aspects of raw material quality be considered, namely the polydispersity of the polymer (which depends on the MW) and the chemical stability of the chosen linker structure. The latter aspect is likely similar to what is required of commercialized PEG derivatives. Based on the natural origin of the polymer, high polydispersity (up to 4.5) is to be expected for commercially available HES-based plasma volume expanders [99]. However, by polymer fractionation, it is possible to obtain HES fractions with much narrower size distributions for further derivatization steps. Such fractions, with a much lower polydispersity of 1.3, were used to form HESylated anakinra from a chosen HES derivative [115]. For HESylated erythropoietin mimetic peptide, HES200/0.5 was fractionated to a size distribution of 130 ± 20 kDa in molecular weight, leading to greater homogeneity in the resulting conjugate [116]. In conclusion, a library of different activated HES derivatives with a variety of linker structures is available in pharmaceutical grade, wherein aspects like polydispersity are drastically improved.

1.6.5. Activity

Polymer conjugation to proteins is known to reduce the specific activity of the protein due to steric hindrance, which inhibits the interaction between the protein and its intended receptor. For example, the PEGylated form of interferon α -2a has a residual activity of just 7% that of the unmodified protein [118]. Therefore, it can be supposed that HESylation will also decrease the conjugate activity in comparison to native protein. Site-specific conjugation, however, can greatly improve activity and binding affinity, as it can reduce the interference in protein–receptor interactions. HESylation of anakinra lowered the initial *in vitro* binding affinity from $k_D = 0.05$ nM (unmodified protein) to 0.32 nM (conjugate) [115]. However, the impaired residual activity was more than compensated for by a 6.5-fold longer half-life and a 45-fold increase in AUC [115]. For the case of HESylated versions of EPO, the selective attachment of the glycosylation site or to the N-terminus resulted in conjugates with a residual *in vitro* activity of approximately 20-40% of the activity of the unmodified EPO standard [98]. A common EPO efficiency marker (hematocrit) showed that the conjugate's PK profiles

were greatly improved upon HES coupling and comparable in both *in vitro* and *in vivo* performance to its marketed PEGylated counterpart (Mircera[®]) [98].

1.6.6. Toxicity

From a toxicological point of view, HES has been reported as safe since its first launch in the US in the 1970s. In September 2013, all intravenous HES products were associated with an increased risk of kidney injury and mortality especially for patients with sepsis, burn victims or the critically ill [119]. Over 40 years the potential immunological risk has been deemed clinically insignificant and is considerably lower than that of dextran or albumin. This is believed to be due to the structural similarity of HES and glycogen, which also contains a branched glucose polymer backbone [120, 121]. In June 2013, the European Medicines Agency's Pharmacovigilance Risk Assessment Committee (PRAC) recommended suspending marketing authorizations for infusion solutions containing hydroxyethyl starch. The German Federal Institute for Drugs and Medical Devices (BfArM) triggered this review based on three recent studies [122-124]. The purpose of these studies was to compare HES as a plasma volume expander with crystalloids in a population of critically ill patients. Patients with sepsis showed an increased risk of kidney injury that required dialysis. Additionally, Perner and Brunkhorst found a higher risk of mortality for patients treated with HES [122, 123]. Presently, HES solutions can be used in patients for the treatment of hypovolaemia caused by acute loss of blood, where infusion solutions based on crystalloids are considered to be insufficient. Treatment regimens with HES solutions should not exceed 24 hours and patient kidney function must be monitored [119].

Despite these hazards, the use of HES for HESylation of proteins and peptides will result in significantly lower doses of HES in the final conjugate when compared to PVEs. Even so, routinely performed toxicity studies must be evaluated to ensure the HESylated conjugate exhibits a satisfactory safety profile.

1.6.7. Effect on protein stability

Prior to our studies, little was known about the effect of HESylation on the stability of the modified protein. We recently showed for HES-anakinra that site-specific HESylation can drastically improve protein stability by increasing the thermodynamic stability and reducing the tendency toward aggregation [115]. The HESylated protein exhibited an increase in melt-

ing temperature of 4.5 K, an enhanced melting enthalpy and possible refolding upon cooling, an effect which could not be observed for the unmodified protein.

1.6.8. Effect on viscosity

Generally, the development of highly concentrated protein solutions is associated with many analytical and formulation challenges [125]. Besides a higher tendency towards aggregation due to molecular crowding at high concentrations, proteins are liable to reversibly self-associate under crowded conditions, leading to an increase in viscosity. The effect on viscosity is dramatically enhanced for polymer-protein conjugates due to the presence of the polymeric component, which tends to entangle at high concentrations. However, highly concentrated formulations are still common, especially for repeatedly dosed protein drugs which are self-administrated by subcutaneous injection in the chronically ill. In the literature, very little is written about the formulation of concentrated solutions of polymer-protein conjugates. It can be inferred that the stiff and branched architecture of HES will be less prone to polymer chain entanglement, acting as more of a hard sphere, than flexible, chain-like polymers such as PEG [99], which worked well in case of highly concentrated PEG- and HESylated anakinra [126].

1.6.9. Lyophilization

HES has as long history of use as a cryoprotectant for red blood cells and human tissue [127-129]. In freeze-drying, frozen solutions of HES have a relatively high glass transition temperature compared to that of disaccharides like trehalose or sucrose. In addition, HES shows excellent glass-forming properties, characterized by solid cakes with high Tgs; it also serves as a Tg-modifying agent [130]. The use of HES as bulking agent and/or lyoprotectant unfortunately failed in most of the reported trials of freeze-dried biologics due to its high molecular weight and its inability to form sufficient hydrogen bonds with the protein [131]. Garzon-Rodriguez *et al.* reported that when used in combination with disaccharides, HES (MW = 200 kDa) enabled greater stability of freeze-dried IL-11 compared to formulations which included only disaccharides or only HES. The formulations which included HES showed higher collapse temperatures and higher glass transition points, leading to improved storage stability and a potentially more economic drying process [132]. However, the effect of HESylation, lyophilization and subsequent storage on protein stability has not been described until now,

nor has the question been answered of whether or not the remarkable properties of HES will be transferred to the HES conjugate during freeze-drying.

1.7. Polysialylation

Polysialic acid (PSA), also known as columinic acid (CA), is a naturally occurring biodegradable polysaccharide and can be used for HLE of biopharmaceuticals. This homopolymer is composed of α -2,8-linked 5-N-acetylneuraminic acid (Neu5Ac) and was discovered on the capsule of neuroinvasive bacteria. The natural function of PSA is to provide a hydrophilic stealth coat for various bacteria. A bacterial surface thus coated can avoid host complement activation and defensive phagocytosis when entering the human body [133]. PSA coating enables organisms to evade the host immune response because PSA is already prevalent in the human body [134]. For example, PSA is known to coat neurons and is involved in nervous system development and repair [135]. When PSA is covalently linked to proteins, the extreme hydrophilicity of the polymer increases the conjugate's hydrodynamic size and provides a protective coating against catabolic enzymes, opsonins, neutralizing antibodies and receptors on phagocytic cells, thus prolonging the presence of the conjugate in the blood stream [136]. Xenetic Bioscience is already using a modified CA for coupling to therapeutic molecules. In order to be used for coupling, the non-reducing end must be either cleaved by periodate oxidation to form an aldehyde group for direct coupling by reductive amination or attachment of another activated linker [136]. Recent publications describing polysialylated insulin using a 22 and 39 kDa PSA showed a threefold increase in the hypoglycemic effect compared to unmodified insulin [136]. Similar approaches were achieved for Fab fragments or a modified antitumor single chain Fv region (scFv), which both experienced increased half-life, reduced immunogenicity and improved tumor uptake [137, 138]. As another example, polysialylation of asparaginase provided an effective coating against proteolytic enzymes, improved its pharmacokinetic profile and decreased protein immunogenicity depending on the degree of coupling of PSA chains [139, 140].

1.8. Recombinant PEG mimetics

1.8.1. PASylation

The term 'recombinant PEG mimetics' describes genetic engineering of large polypeptide sequences containing a number of selected amino acids. Fusion of this polypeptide with pro-

teins and peptides increases their hydrodynamic volume and provides a completely biodegradable conjugate. XL-Protein GmbH, in one of the first approaches, fused a PEG mimetic based on the amino acids proline, alanine and serine (PAS) to a number of therapeutic proteins [141]. The concept is to maintain PEG-like properties in an engineered, recombinantly produced polypeptide, which should include a large hydrodynamic size, the absence of positive or negative charges in the polymer backbone, high aqueous solubility and a random coiled structure. Therefore, hydrophobic and charged amino acid side chains were not considered. Carboxamide-containing side chains from Asn and Gln were also eliminated due to their known tendency toward aggregation as well as their role in protein folding pathologies such as Huntington's disease [142]. Threonine, a β -branched amino acid, was also excluded due to its pronounced propensity for β -sheet formation. Histidine was not considered because of the high binding affinity of the imidazole side chain for metal ions, as well as its basic character. Due to poor solubility with increasing peptide length and its compacting effect on a random coiled and unfolded polypeptide chain, glycine was also excluded [143]. The final PAS molecule is a flexible and uncharged polypeptide chain and can range in length from 200 to 600 amino acid residues; the neutral charge does not provoke changes in the isoelectric point of the molecule of interest upon fusion. The well-defined amino acid sequence tends to form a random coiled structure, which is highly dependent on the length of the polypeptide and can be assessed by CD-spectroscopy [144]. A PAS 600 amino acids in length is characterized by a MW of 50 kDa and appears as a 0.5 MDa molecule in analytical size exclusion chromatography [145]. The PASylated molecule can experience a 10- to 100-fold increase in circulation time depending on the length of the chosen polypeptide. PASylation also enables conjugate production in an *E. coli*-based cell line, avoiding the use of mammalian or CHO cells. The PAS molecule itself is believed to be eliminated by intracellular enzymatic digestion but is highly resistant to serum proteases [145]. PASylation of three relevant protein therapeutics (hGH, IFN α -2b and a recombinant Fab fragment of the humanized anti-HER2 antibody 4D5 (trastuzumab) proved that PAS extends half-life by retarding conjugate renal clearance [144]. In addition, the modified proteins showed higher thermal resistance, observed as higher melting points, while retaining their native charge and high *in vitro* binding affinity [144].

1.8.2. XTENylation

The XTEN technology from Amunix Inc. is a second approach to producing genetic PEG mimetics. The polypeptide sequence is based on alanine, glutamic acid, glycine, proline, serine and threonine. The exclusion of hydrophobic amino acids like phenylalanine, isoleucine,

leucine, methionine, valine, tryptophan and tyrosine promotes a maximal hydrodynamic radius and avoids generation of a compact polypeptide structure, which can induce protein aggregation. From the immunological point of view, the hydrophobicity of such amino acids is considered to play a critical role in polypeptide recognition by the immune system when the reaction is driven by binding to MHC receptors [146]. The negatively charged amino acids in the sequence provide for an unstructured chain with strong intramolecular repulsion. This is necessary both to prevent chain collapse and to produce a highly flexible polymer backbone. The molecule shows high resistance to plasma proteases and remains intact in the bloodstream for a significant period of time [147]. Degradation is driven mainly by internalization into cells and subsequent rapid destruction. Therefore, it can be supposed that long-term intracellular accumulation will be decreased [147]. In contrast to PASylation, XTENylation involves a conscious decision to make the overall net charge of the molecule negative after coupling. Adding polypeptide sequences of 864 amino acids based on ala (72 aa), glu (144 aa), gly (144 aa), pro (144 aa), ser (216 aa) and thr (144 aa) to exenatide resulted in a 125-fold increase in half-life and a depot effect upon s.c. injection [146]. The lack of immunogenic potential has been proven in mice and rabbits, which were treated by weekly injection of the conjugate over a period of 6 weeks. However, XTENylation can lower the bioactivity of the conjugate relative to the native protein. In the case of XTENylated glucagon, activity decreased to 15% that of the native form [148]. Additionally, the modification of human growth hormone (VRS317 with a MW = 119 kDa) showed a 12-fold reduction in *in vitro* potency due to significantly attenuated receptor recognition, caused primarily by the highly negatively charged surface of the conjugate [149]. However, XTENylation yielded a net positive benefit due to reduced receptor-mediated clearance and a reduction in side effects, such as lipoatrophy at the injection site, observed for other long-acting derivatives like PEGylated hGH [149, 150]. This novel construct caused a sustained pharmacodynamic response over the course of one month, an effect which promises to increase patient compliance and reduce possible side effects of repeated dosing [149]. XTENylation can also improve the physicochemical properties of hydrophobic peptides like glucagon, resulting in improved hydrophilicity as well as improved stability in liquid formulation. Improved colloidal stability could enable formulators to avoid an additional lyophilization step recommended for the native form of glucagon [148].

1.9. Aims of the thesis

Many clinically relevant improvements have been achieved by grafting polyethylene glycol to proteins and peptides; this strategy has resulted in more than ten approved PEGylated drugs. However, PEGylation suffers from a number of shortcomings. These shortcomings inspired an investigation, in collaboration with Fresenius Kabi, into whether or not some of these drawbacks might be overcome by protein HESylation while preserving the favorable physicochemical properties enabled by PEGylation.

Chapter II describes the effect of HESylation[®] on the model protein anakinra. A battery of analytical methods is used to characterize the conjugate's physicochemical properties, such as conformational and colloidal stability. These analytical results are supported by pharmacokinetic results from *in vivo* experiments.

Little is known about the effect of polymer conjugation on APIs intended for use in highly concentrated formulations. Therefore, the aim of chapter III is to use anakinra as a model protein for comparing HESylation and PEGylation regarding their effect on the physicochemical characteristics of the protein, as well as its formulation and stability in the challenging situation of highly concentrated protein solutions. To wit, the influence on viscosity, thermodynamic stability and storage stability are explored.

Finally, chapters IV and V are dedicated to an evaluation of the stability of two PEGylated and HESylated model proteins upon freeze-drying and subsequent storage at elevated temperatures. Lyophilization of PEGylated proteins still represents a challenge, mainly due to the high tendency of PEG to crystallize during freeze-drying. Thus, its impact on storage stability at elevated temperatures in the presence/absence of lyoprotectants is the main focus of these studies. Finally, the influence of HESylation on protein stability upon lyophilization is evaluated, for both dilute and highly concentrated protein samples.

1.10. References

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Chapter II – Half-life extension through HESylation

2. Protein HESylation for half-life extension: Synthesis, characterization and pharmacokinetics of HESylated anakinra

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Abstract

Half-life extension (HLE) is becoming an essential component of the industrial development of small-sized therapeutic peptides and proteins. HESylation[®] is a HLE technology based on coupling drug molecules to the biodegradable hydroxyethyl starch (HES). In this study, we report on the synthesis, characterization and pharmacokinetics of HESylated anakinra, where anakinra was conjugated to propionaldehyde-HES using reductive amination, leading to a monoHESylated protein. Characterization using size exclusion chromatography and dynamic light scattering confirmed conjugation and the increase in molecular size, while fourier transform infrared spectroscopy showed that the secondary structure of the conjugate was not affected by coupling. Meanwhile, microcalorimetry and aggregation studies showed a significant increase in protein stability. Surface plasmon resonance and microscale thermophoresis showed that the conjugate retained its nanomolar affinity, and finally, the pharmacokinetics of the HESylated protein exhibited a 6.5-fold increase in the half-life, and a 45-fold increase in the AUC. These results indicate that HESylation[®] is a promising HLE technology.

Keywords

HESylation[®], anakinra, microcalorimetry, binding affinity, pharmacokinetics

2.1. Introduction

Since the recombinant DNA revolution in the 80s, there has been a constant increase in the development of new protein therapies [1]. Except for monoclonal antibodies (MAbs), many of the marketed and investigational biopharmaceuticals have a molecular size below the renal clearance threshold (i.e. < 60 kDa), and are thus rapidly eliminated through the kidneys (such as cytokines, growth factors, antibody fragments or protein scaffolds) [2]. This requires frequent injection and is associated with reduced compliance. The extension of a protein's half-life can be achieved by the covalent coupling of water soluble polymers, such as polyethylene glycol (PEG), as pioneered by Davies and Abuchowsky in the 70's [3, 4]. Such covalent attachment of hydrophilic polymers can have the added advantages of increasing protein solubility, enhancing stability, reducing proteolysis and immunogenicity [5, 6].

PEG, however, is not biodegradable, raising concerns about possible vacuolization of the kidney or the liver upon chronic administration of high doses of PEGylated proteins [7-9]. This and other limitations have spurred activity to find other half-life extension (HLE) technologies, with many being pursued industrially and academically [10, 11]. Among these, HESylation[®] represents a promising HLE strategy [12]. The latter involves the covalent coupling of hydroxyethyl starch (HES) to a biopharmaceutical to increase its size. HES is highly biocompatible and biodegradable, and is clinically approved as one of the first line plasma volume expanders (PVEs), with applied doses up to 200 g/day, making it an attractive hydrophilic polymer for HLE [13-15].

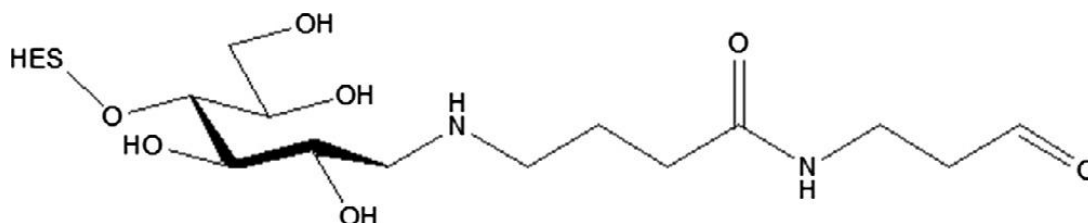
In this work, we report on the synthesis, characterization and pharmacokinetics of a model HESylated protein, namely recombinant human interleukin 1 receptor antagonist (rhIL-1ra, also known as anakinra). Anakinra is a 17.26 kDa protein that binds to IL-1 receptor, blocking the inflammatory action of IL-1, and is thus approved for adult patients with rheumatic arthritis who show an inadequate response to other disease modifying anti-rheumatic drugs (DMARDs) [16]. Due to its short half-life [16], anakinra has to be administrated by a daily injection of 100 mg, making it an ideal candidate for HLE. Accordingly, the aim of this study is to synthesize and purify HESylated anakinra, compare its physicochemical properties to the native protein, and finally investigate the binding affinity and pharmacokinetic properties of the modified protein.

2.2. Experimental procedures

2.2.1. Materials

Kineret[®] was obtained from SOBI (Stockholm, Sweden). Activated hydroxyethyl starch (HES, with a weight average molar mass (M_w) of approximately 85 kDa, number average molar mass (M_n) ~ 65 kDa, and a polydispersity of 1.3). was from Fresenius Kabi Deutschland GmbH (Bad Homburg, Germany). The activated HES carries an aldehyde linker at its reducing end group as shown in **scheme 1**. All other chemicals were analytical grade and used as obtained.

Scheme 1. Activated HES carrying an aldehyde linker at its reducing end.



2.2.2. Synthesis and purification of HESylated anakinra

In a 1 L glass reaction vessel equipped with a thermostated jacket and a blade stirrer, 2.5 g protein (19 mL; 134 mg/mL) were diluted into 220 mL 0.1 sodium acetate buffer (pH = 5.0) pre-chilled to 5°C. Hydroxyethyl starch carrying a reactive propionaldehyde group was dissolved in the same buffer to yield a 40% (w/v) solution and 75 mL of the HES solution (representing a 3:1 molar ratio of polymer to protein) were combined with the protein under moderate stirring. The reductive amination reaction was started by addition of 12.5 mL of a freshly prepared 0.5 M NaCNBH₃ solution in water and incubated overnight (18 h) under moderate stirring at 5°C.

The conjugate was purified by anion-exchange chromatography using Q Sepharose HP material and an Äkta Purifier 100 chromatography system (both GE Healthcare, Munich, Germany). The system was operated at room temperature with a typical flow rate of 10 mL/min. The column (XK 16/20) was first equilibrated with 5 CV of eluent A (10 mM Tris acetate, pH = 8.0). The reductive amination reaction mixture was 10 fold diluted with eluent A, the pH adjusted to pH = 8.0 using NH₄OH solution and beta-mercaptoethanol added to a final concentration of 1 mM to destroy potential disulfide-mediated protein dimers. Up to 800 mg of conjugate were then loaded onto the column at a flow rate of 10 mL/min, followed by a washing

step with 2 CV 5% eluent B (eluent A + 250 mM NaCl) to remove the unbound, excessive HES polymer. The conjugate was eluted in a step gradient at 25% eluent B for 4 CV. Peak fractions were collected and subsequently a buffer exchange to CSE (10 mM citrate, 140 mM sodium chloride, 0.5 mM EDTA, pH = 6.5) buffer w/o Tween 80 and up-concentration performed by TFF using a 50 cm² PES (polyethersulfone) membrane capsule with a MWCO of 10 kDa operated on a MinimateTM benchtop system (both from Pall, Dreieich, Germany). The retentate of the TFF step was subjected to a 0.22 µm filtration step, aliquots of the filtered solution shock-frozen in liquid nitrogen and stored at -80°C until further use.

2.2.3. Determination of conjugate concentration

Concentration determination was performed by UV spectroscopy using the extinction coefficient at 280 nm with an extinction coefficient of 13392 M⁻¹cm⁻¹ corrected for the formulation buffer and potential stray light contribution at 320 nm [17].

2.2.4. SEC-MALLS measurement for the determination of the molar mass and size of the conjugates

The molar mass and size of native and HESylated anakinra were investigated by a size exclusion chromatography-multi angle laser light scattering (SEC-MALLS) on the AF2000 Focus (Postnova Analytics, Landsberg/Lech, Germany) equipped with a multi-angle laser light scattering (MALLS) miniDAWN Tristar detector (Wyatt Technology, Dernbach, Germany), refractive index detector (PN 3150, Postnova Analytics, Landsberg/Lech, Germany) and a Shimadzu SPD-10A UV-VIS Detector (Shimadzu, Duisburg, Germany). For SEC separation, a Superose 6 10/300 GL column (GE Healthcare, Uppsala, Sweden) was used with CSE buffer as the running phase including 0.02% sodium azide at a flow rate of 0.5 mL/min over 45 min. Each run was performed by the injection of 50 µg, based on the protein weight. The data were analyzed by the Astra software version 5.0 (Wyatt Technology, Dernbach, Germany), where the rms (root mean square) radii were calculated from the MALLS data. The protein conjugate analyses were performed by the Wyatt protein conjugate application embedded in the Astra 5.0 software. UV extinction coefficient for anakinra is 13392 M⁻¹cm⁻¹ [17]; dn/dc is 0.1850 mL/g for anakinra and 0.1460 mL/g for HES [18].

2.2.5. Dynamic light scattering (DLS)

The hydrodynamic radius was measured by DLS using the Malvern Zetasizer (Malvern Instruments, Herrenberg, Germany). Native and HESylated anakinra were diluted to a final concentration of 1 mg/mL (based on the protein). Before scanning, all samples were filtered through a 0.2 μm filter. The hydrodynamic radius is expressed as the Z- average, together with the polydispersity index (PI) as an indication for the breadth of size distribution.

2.2.6. Fourier transform infrared spectroscopy

FTIR spectroscopy experiments were performed using the Bruker Tensor 27 FTIR with the Bruker AquaSpec Cell (Bruker Optics, Ettlingen, Germany). The sensor was cooled with liquid nitrogen and a constant gaseous nitrogen flow. The samples were analyzed at a concentration of 3 mg/mL (based on the protein part) in 240 scans against CSE buffer which was used for background subtraction. Using the OPUS Software, the second derivative of the spectrum for each sample was obtained in the range of 1600 to 1700 cm^{-1} . The curves were normalized by vector normalization.

2.2.7. Surface plasmon resonance measurements for *in vitro* binding affinity

The binding affinity was analyzed by surface plasmon resonance (SPR) using a Biacore T100 instrument (Biacore AB, Uppsala, Sweden). The recombinant IL-1 receptor type I (R&D Systems, Wiesbaden, Germany) was immobilized by standard EDC/ NHS coupling on a CM3 chip in acetate buffer ($c_{\text{acetate}} = 10 \text{ mM}$; $\text{pH} = 4.5$) to final response of 569 RU. A kinetic study was performed for native and HESylated anakinra at room temperature with a constant flow rate of 30 $\mu\text{L}/\text{min}$ and 180 s for the on-rate and 900 s for the off-rate, after which, the receptor was regenerated with 50 mM NaOH for 5 s. Different sample concentrations were produced by dilution in HBS-EP running buffer (contains HEPES 10 mM, sodium chloride 150 mM, EDTA 3 mM and P-20 0.05%). The binding data were subtracted from those obtained by running the ligands on a control cell lacking the bound receptor, and analyzed for kinetic and affinity characteristics using the Biacore T200 Evaluation Software. The curves were fitted by non-linear regression according to the Langmuir binding isotherm using the 1:1 model.

The specificity of the interaction was confirmed by lysozyme, which was allowed to interact with the receptor using the same set up, but did not show any binding with the receptor (data not shown).

2.2.8. Microscale Thermophoresis (MST) for *in vitro* binding affinity

MST measurements were performed on a Monolith NT 115 (NanoTemper Technologies, Munich, Germany). The IL-1 receptor type I was labeled with NT Dye 495 using a NanoTemper standard labeling protocol. The receptor was first rebuffered in the labeling buffer to a final concentration of 20 μ M. NT Dye 495 was added in threefold molar excess and incubated for 30 min at room temperature. Finally, the labeled protein was separated from excess dye by a gravity flow column and concurrently rebuffered in the same buffer used for the SPR measurements, namely HBS-EP (contains HEPES 10 mM, sodium chloride 150 mM, EDTA 3 mM and P-20 0.05%). For MST measurements, native and HESylated anakinra were diluted in 16 dilution steps in HBS-EP covering the range from 1 μ M to 0.03 nM. Then 2 μ l of 100 nM labeled receptor were added to 20 μ l sample solution. The samples were loaded in NT hydrophobic capillaries (NanoTemper Technologies, Munich, Germany) and measured with a laser power of 40%, with a laser on- rate of 30 s and a laser off- rate of 5 s in triplicates. The kD values of native and HESylated anakinra were calculated by the NanoTemper software version 1.0.1.

2.2.9. Microcalorimetry

Differential scanning calorimetry (DSC) was performed using a MicroCal VP-DSC (MicroCal Inc, Northampton, MA). The samples were diluted to a final concentration of 1 mg/mL (based on protein) and measured against CSE buffer in 2 consecutive scans (i.e. heating, cooling, then 2nd heating) to investigate the melting temperature and unfolding reversibility. Each heating run was performed in the range of 20 to 95°C with a heating rate of 1°C/min. The thermograms were analyzed using the Microcal Origin Software. The melting temperature was calculated as the maximum of heat capacity. The ratio of the enthalpies of both 1st and 2nd heating runs is expressed as the unfolding reversibility in %. All measurements were performed in triplicates.

2.2.10. Thermal stability

The thermal stability of native and HESylated anakinra was tested using the method of Raibekas *et al.* [17]. 180 μ L Protein samples at a concentration of 25 mg/mL (related to protein) were placed in a flat-bottom 96 well plate (Greiner Bio-One GmbH, Frickenhausen, Germany). The plate was incubated at 40°C in a FLUOstar Omega plate reader (BMG Labtech; Ortenberg; Germany) for a period of 500 min. The aggregation process was followed by the increase in optical density at 350 nm. After each reading step the samples were shaken with 700 rpm for 3 s.

2.2.11. Pharmacokinetic study

Male Wistar rats (250 – 300 g body weight) were randomly allocated to two test groups of six animals each. Anakinra and the molar equivalent amount of HESylated anakinra dissolved at 2.5 mg/mL in CSE-buffer (1.93 mg/mL NaCitrate, 8.2 mg/mL NaCl, 0.18 mg/mL Na-EDTA, pH = 6.5) were intravenously administered at a single dose of 5 mg/kg. Within each group, rats were divided into two subgroups of three animals which were bled at alternating time points to avoid exceeding recommended volume limits for non-terminal blood withdrawal. Blood samples (approx. 200 μ L each) were withdrawn from tail vein of the animals of both groups at predetermined time points. The collected blood was immediately transferred into lithium heparin-containing Microvette[®] tubes, shaken by hand and stored for 60 min at most on crushed ice until centrifugation (10000 x g and 4°C for 10 min). After centrifugation, the supernatant (plasma) of each sample was collected and immediately frozen.

The plasma concentrations were analyzed by a commercial enzyme linked immunosorbent assay (ELISA) kit method (RayBio[®] Human IL-1ra ELISA Kit) with anakinra and HESylated anakinra as standards after appropriate dilution. The assay was used as described in the user manual of the ELISA manufacturer (incubation of calibration standards and study plasma samples was done overnight at 4°C). Plasma concentration data are expressed as mean \pm SD. The pharmacokinetic parameters were calculated from the plasma concentrations after administration (noncompartmental analysis, AUC calculated using linear trapezoidal method) using PKSolver 2.0 [19].

2.3. Results

Anakinra was coupled to HES at the N-terminus by a reductive amination reaction through a single terminal aldehyde group on HES. After anion exchange chromatography, and re-buffering into citrate buffer pH = 6.5, a highly purified mono-HESylated protein was obtained, with a yield of 65%.

2.3.1. Molar mass and size

The molar mass and size of the obtained mono-conjugate were characterized using SEC-MALLS as well as DLS, respectively. The combination of SEC for molecular separation with MALLS, UV and RI for detection is a powerful method for an exact calculation of the molar mass as well as the quantification of the extent of coupling and/or aggregation of the conjugated proteins. Results show that the weight average molar mass (M_w) of the native anakinra, calculated by MALLS and UV detection, was 16.6 kDa, which is in good agreement with the theoretical protein mass of 17.26 kDa (**Figure 1 and Table 1**). The HESylated protein (**Figure 1 bottom**) showed a M_w of 105.5 kDa, which is also quite close to the hypothetical mass of 102.7 kDa.

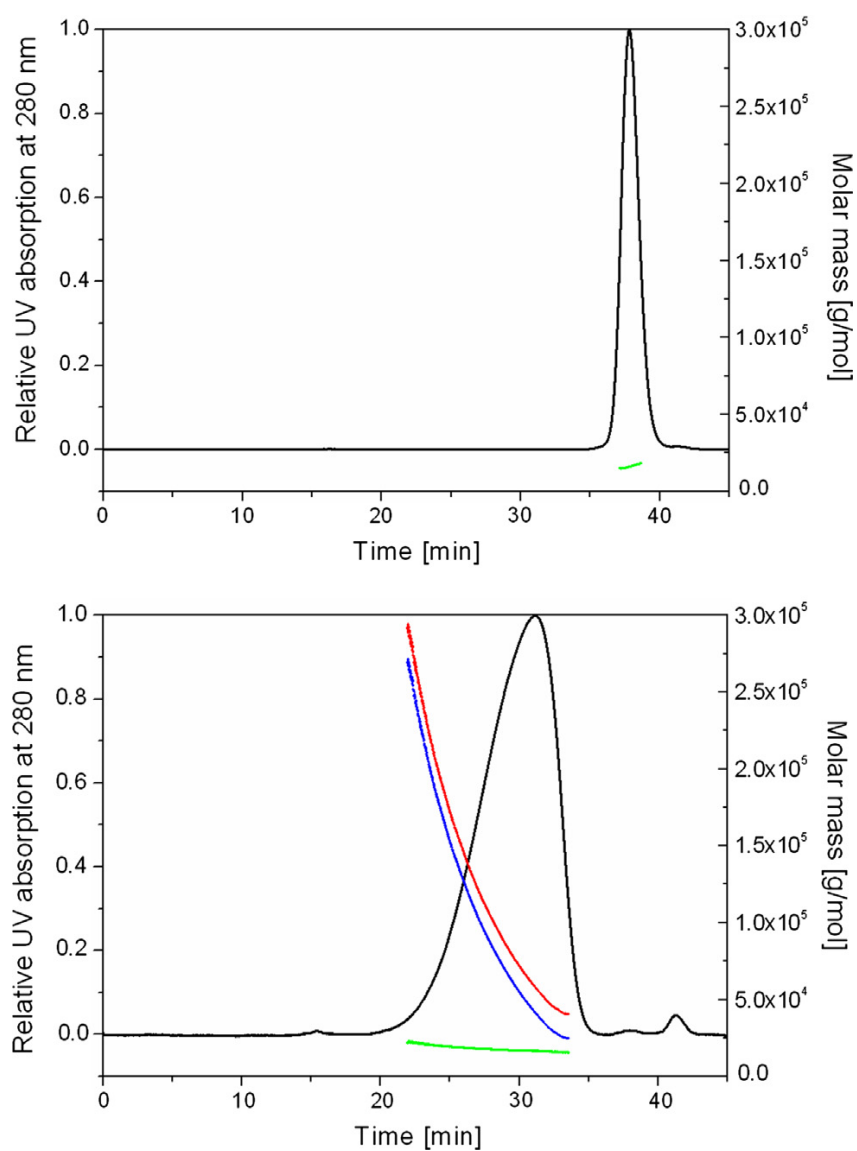


Figure 1: SEC-MALLS chromatograms for determining the molar mass of native (top), and HESylated anakinra (bottom). The molar mass distributions of the conjugate, the protein part and the polymer part are expressed in red, green and blue lines, respectively.

Table 1: Weight average molar mass (M_w) and polydispersity index (PDI) for activated HES, native and HESylated anakinra.

Sample	M_w [kDa]	Polydispersity	Hydrodynamic diameter [nm] ^b	PDI ^b
Activated HES	85	1.3	-	-
Native anakinra	16.6 ^a	1.00 ^a	4.36 ± 0.18	0.16 ± 0.03
HESylated anakinra	105.5 ^a	1.24 ^a	14.73 ± 0.25	0.20 ± 0.01

^a determined by SEC-MALLS

^b determined by DLS

The hydrodynamic size of the conjugate was determined using DLS as seen in **Table 1**. The size of anakinra is 4.36 nm, which is quite comparable to the literature-reported value [6]. Meanwhile, the size of HES-conjugate is nearly three times that of native anakinra as seen in **Table 1**.

2.3.2. Protein conformation

The effect of HESylation[®] on anakinra's conformation was investigated using FTIR. The 2nd derivative spectrum of anakinra in **Figure 2** shows the main characteristics of its secondary structure, namely a predominant beta sheet arrangement (peak at 1640 cm⁻¹) and beta-turn structures (peak at 1685 cm⁻¹) [20]. The spectrum of HESylated anakinra nearly superimposes with that of the native protein, showing that HESylation[®] did not lead to significant changes in anakinra's secondary structure.

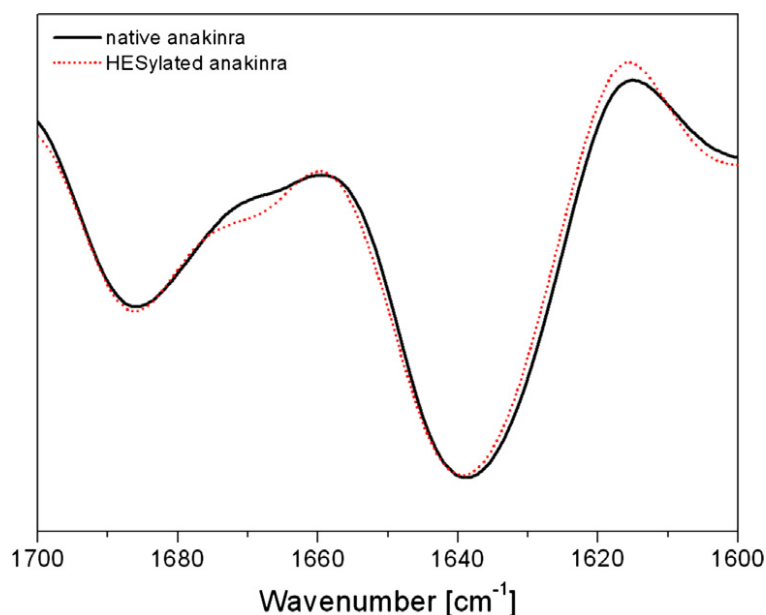


Figure 2: Second- derivative amide I FTIR spectra of native anakinra (black solid line), and HESylated anakinra (red dotted line).

2.3.3. Microcalorimetry and thermal stability

Microcalorimetric measurements show that anakinra has a melting temperature of $58.0 \pm 0.5^{\circ}\text{C}$, which is in agreement with the previously reported value of 56°C [21], whereas HESylated anakinra shows a 4.8 K increase in the melting point in comparison to the native protein (**Figure 3 and Table 2**). In addition to the observed increase in T_m , the enthalpy of melting increases significantly upon polymer conjugation (**Table 2**). Furthermore, the protein conjugate showed a high degree of refolding upon cooling (90%), contrary to the native protein, which precipitated upon denaturation (**Figure 3 and Table 2**). Meanwhile, the stress stability study performed in a microwell-plate at 40°C (**Figure 4**) showed that native anakinra aggregated rapidly as manifested by a rapid increase in turbidity after less than 30 min, similar to previously reported results [17], while the HESylated anakinra did not show an increase in turbidity over several hours.

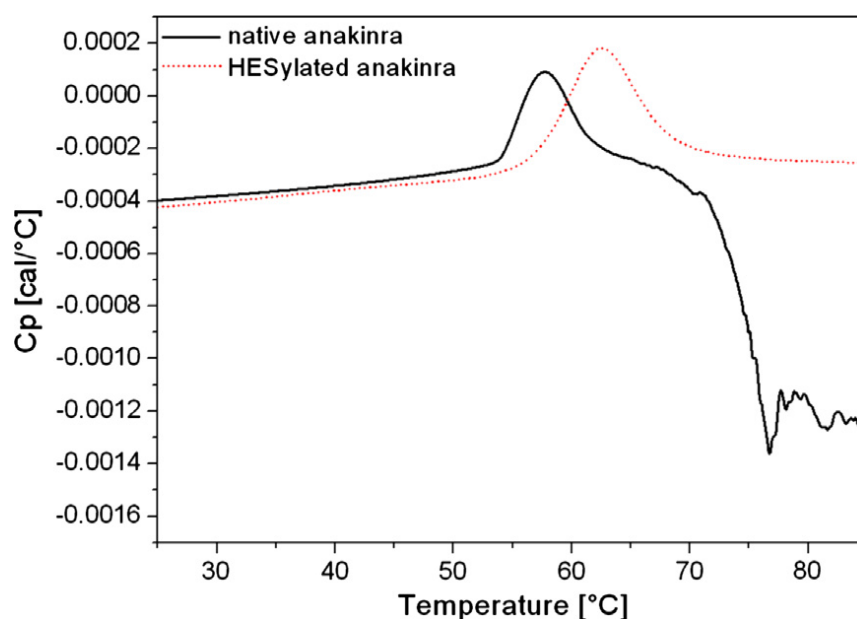


Figure 3: Microcalorimetry thermograms of native anakinra (black solid line) and HESylated anakinra (red dotted line). Native anakinra aggregated directly after unfolding, leading to the observed decrease in heat capacity, while HESylated anakinra did not.

Table 2: Thermodynamic properties of native and HESylated anakinra

Sample	T_m [°C]	ΔH [kcal/ mole]	Δcp [kcal/mole/°C]	Unfolding re- versibility [%]
Native anakinra	58.0 ± 0.5	58.4 ± 0.1	11.7 ± 0.4	0
HESylated anakinra	62.8 ± 0.3	100.8 ± 1.0	19.4 ± 0.3	90.3 ± 0.9

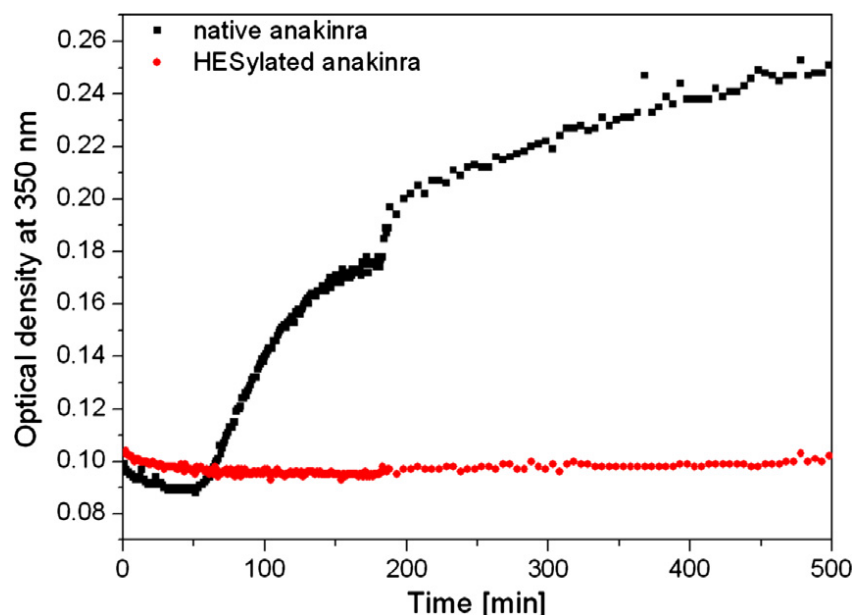


Figure 4: Optical density at $\lambda = 350$ nm as a function of time for anakinra (black dots) and HESylated anakinra (red dots) solutions (protein concentration = 25 mg/mL) stored at 40°C for 500 min in a 96 well plate with eventual shaking after each reading step

2.3.4. *In vitro* binding affinity

The binding affinity was measured using SPR and MST. In case of SPR measurements, the recombinant IL-1 receptor type I was immobilized with a low density to minimize mass transport and rebinding effects. **Table 3** illustrates the k_D values of native and HESylated anakinra including the on- and off-rates. The wild type protein showed the fastest on-rate followed by a very slow off-rate (**Figure 5 and Table 3**), which led to a k_D value of 0.05 nM. The on-rate of the HESylated anakinra is nearly one order of magnitude lower than the native protein, while the off-rates for both proteins are similar (**Table 3**), leading to a slightly higher dissociation constant ($k_{D_{\text{HESylated anakinra}}} = 0.32$ nM). Meanwhile, MST results show that the native protein had a k_D of 3.85 nM, while the conjugate's k_D was 10.7 nM (**Figure 6 and Table 3**).

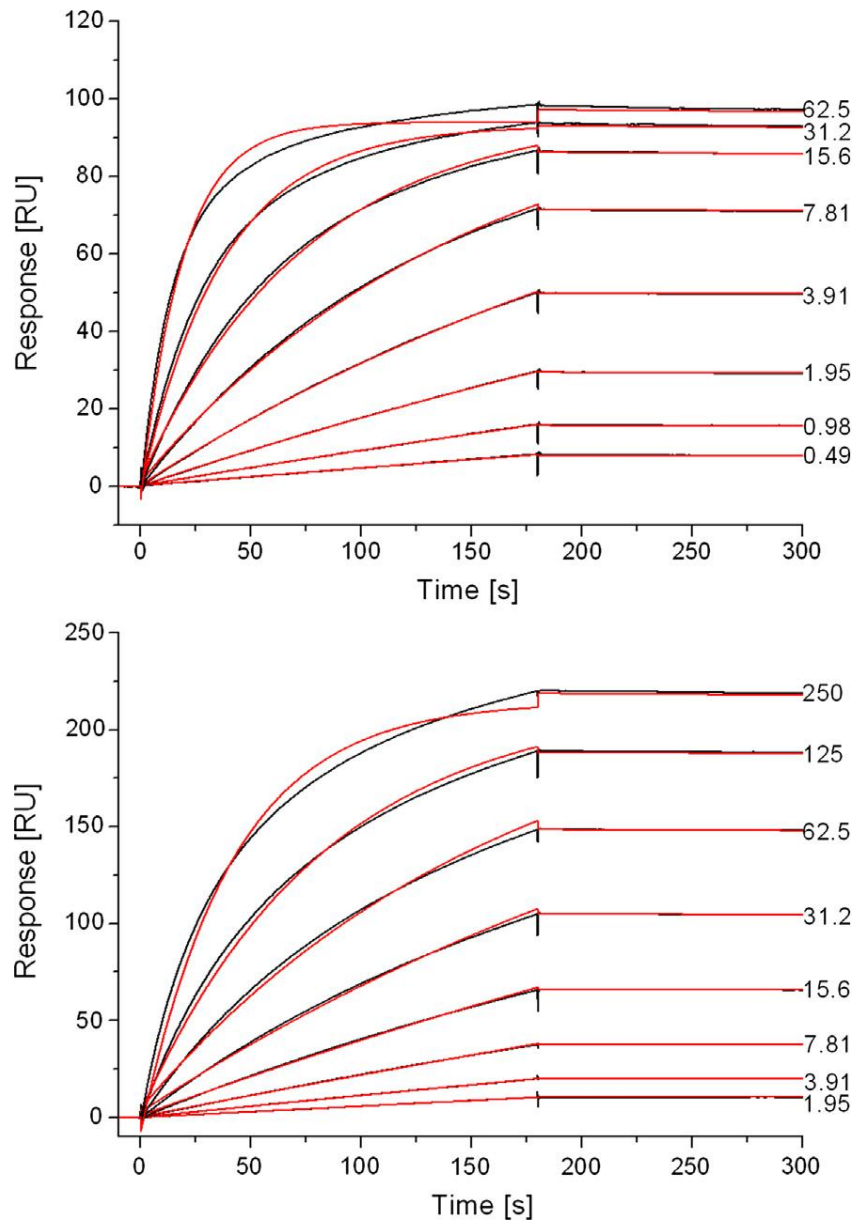


Figure 5: SPR analyses for the binding of native anakinra (top) and HESylated anakinra (bottom) to IL-1 receptor type I. All binding curves (black lines) were fitted by Langmuir (1:1) binding isotherm (red lines).

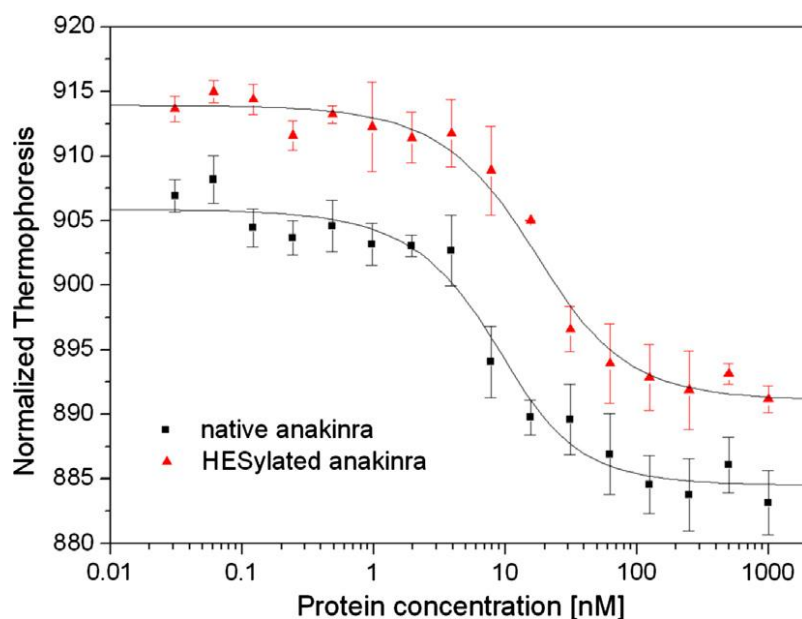


Figure 6: MST measurement for of the binding of native and HESylated anakinra to IL-1 receptor type I as a function of ligand concentration.

Table 3: On- rates, off-rates and dissociation constants for native- and HESylated anakinra from SPR measurements as well as dissociation constant from MST measurements

Sample	SPR			MST
	k_{on}	k_{off}	k_D	k_D
	[1/Ms]	[1/s]	[nM]	[nM]
Native anakinra	8.54E+5	3.91E-5	0.05	3.85
HESylated anakinra	9.61E+4	3.11E-5	0.32	10.7

2.3.5. Pharmacokinetic profiles of native and HESylated anakinra

The pharmacokinetic properties of anakinra and HESylated anakinra were evaluated following intravenous administration in male Wistar rats. No toxicity or adverse effects were noted in the treated rats. **Figure 7** illustrates the plasma concentration profiles during a 72 h experimental period, and the pharmacokinetic parameters calculated from the data by noncompartmental analysis are summarized in **Table 4**. **Figure 7** shows that plasma concentrations of native and HESylated anakinra demonstrated an exponential declining pattern after i.v. administration, but the elimination of HESylated anakinra was much slower than that of native

protein. The half-life of HESylated anakinra was increased about 6.5 times, as compared to that of native anakinra. Additionally, HESylated anakinra showed a reduced clearance (CL) and a marked increase (approximately 45 times) in the area under the plasma concentration curve (AUC), as well as a marked decrease in the apparent volume of distribution, indicating higher confinement to a smaller fluid volume, and lesser diffusion out of the blood compartment.

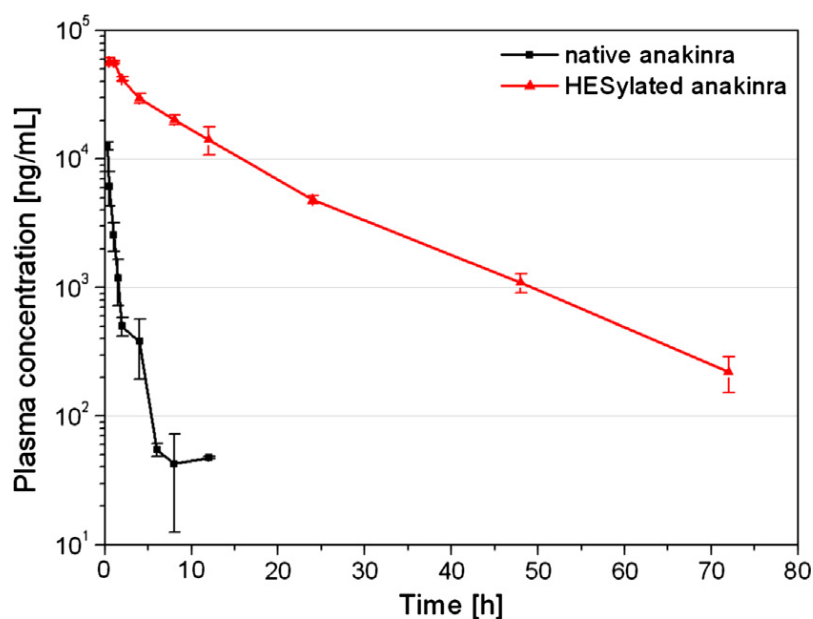


Figure 7: Plasma level of native and HESylated anakinra after single intravenous administration to rats. Closed symbol: anakinra; open symbol: HESylated anakinra. Rats were injected with 5 mg/kg anakinra or molar equivalent amount of HESylated anakinra.

Table 4: Pharmacokinetic parameters after anakinra and HESylated anakinra administration to rats.

Parameter	Native anakinra	HESylated anakinra
AUC (h*ng/mL)	12,287	548,790
$t_{1/2}$ (h)	1.7	10.8
CL (mL/h/kg)	403.3	9.1
V _z (mL/kg)	958	141

Results are expressed as mean ($n = 3$). Abbreviations: AUC, area under the curve; $t_{1/2}$ (h): apparent terminal elimination half-life; CL: observed clearance; V_z : apparent volume of distribution

2.4. Discussion

With the current developments in engineering protein molecules for a multitude of targets and diseases, HLE became an important enabling strategy for many protein development programs. Due to the limitations of PEGylation [22, 23], newer technologies are being pursued, and these can be grouped into 2 broad strategies: 1) physical increase in the size of the molecule of interest above the renal glomerular filtration limit by coupling to biodegradable, water soluble polymers (such as polysialic acid [24, 25]) or fusion to random-coil-forming polypeptides (such as Xten technology [26, 27], or PASylation [28]). The other strategy involves 2) the use of a binding mechanism, such as binding to the Fc neonatal receptor (FcRn) to make use of the FcRn natural recycling mechanism in the endothelial cells [10]. The latter can be achieved by fusion to the Fc part of antibodies, or fusion to serum albumin [10].

In this study, we report on HESylation[®] as a promising HLE technology. It involves coupling of hydroxyethyl starch (HES) to molecules of interest to increase their size and thus their circulation time. HES is a semi-synthetic water-soluble polymer produced by the reaction of starch with ethylene oxide [29]. It is degraded by the action of serum alpha-amylase, and the rate of degradation can be tailored by controlling the extent of hydroxyethylation [30]. Such favorable properties, in addition to its proven safety record and extensive clinical use as plasma volume expander, render HES as an attractive alternative to PEG.

In this study, HESylation[®] was applied to anakinra. The latter is used for the treatment of rheumatic arthritis, but has a terminal half-life of only 108 min in humans [16], thus many researchers have attempted to increase its circulation time and reduce the dosing frequency [31, 32]. Those attempts involved coupling it to PEG, either at the N-terminus [31], or at the cysteine or lysine residues [32]. In the current study, we report a site-specific conjugation of HES at the N-terminus of anakinra using reductive amination. The reaction makes use of a single terminal aldehyde group on the activated HES, which forms a Schiff's base with the protein's N-terminal-amino group, and is subsequently reduced to a secondary amino group. The coupling chemistry is based on the difference in pKa between the ϵ -amino groups of lysine and that of the N-terminal amino group, leading to a preferred reaction with the N-terminus at slightly acidic conditions [33-35]. Previous reports on HESylation[®] described

coupling several peptides or small molecules to one HES chain [14, 36, 37], as well as site-specific conjugation [12].

Despite the putative regioselectivity of the reductive amination reaction described above, it can not be excluded that also certain exposed Lys residues become reactive with the polymer, leading to an inhomogenous multi-HESylated product, especially when a high excess of the modification reagent (i.e. activated HES) is used for a prolonged reaction time. To minimize this risk, the small-scale conjugation process was directed towards maximizing homogeneity of the product rather than yield. After preliminary optimization runs, it was possible to identify conditions that produced predominantly mono-conjugated protein (as followed by SEC analysis, see below), with a coupling efficiency restricted to ~80 %, and an overall process yield of 65% after all purification steps. The predominately regioselective coupling at the N-terminus was confirmed by peptide mapping after digestion by trypsin (Promega, Madison, USA) for 20 h at 37°C, followed by analysis on a Q-Star[®] XL Hybrid LC/MS/MS system. Results showed that, the heptapeptide at the N-terminus of anakinra could not be recovered after HESylation, in contrast to the native protein, which confirms the N-terminal coupling (data not shown).

SEC-MALS and RP-HPLC analysis showed that a predominantly mono-HESylated protein was obtained after synthesis and purification, with a purity of > 98 %, containing less than 0.5% of non conjugated protein, free HES polymer as well as soluble aggregates. Additionally, the calculated masses of the protein and the conjugate agree quite well with the theoretical values. One obvious difference between native anakinra and the HESylated one is the broad molar mass distribution of the latter (PDIHESylated anakinra = 1.244). This is quite close to the PD of the activated HES molecule (= 1.3), and generally due to the natural origin of the polymer, with polydispersities between 1.9 and 4.5 obtained for commercially available plasma volume expanders based on HES [29]. However, by sample fractionation, it is possible to obtain HES fractions with even narrower size distribution compared to the starting material used for the protein conjugation in this study. Meanwhile, DLS results show that the hydrodynamic diameter of the anakinra increased from 4.36 to 14.73 nm for the HESylated protein. This is essential for increasing the half-life, since it is known that molecules bigger than the pores of the glomerular basement membrane (approximately 5.0 nm) have a reduced glomerular filtration rate [38].

The influence of HESylation[®] on the secondary structure of the protein was investigated using FTIR. The vast majority of literature reports support the notion that polymer conjugation to proteins has no effect on a protein's secondary and tertiary structure [39, 40]. For example,

Kinstler *et al.* studied the effect of PEGylation on the secondary structure of GCSF by circular dichroism, and found out that conjugation did not influence the protein conformation [33], a result later confirmed by Rajan *et al.* in an FTIR study [39]. There are however few studies which describe changes in protein conformation upon PEGylation [41, 42]. For instance, Yu and coworkers reported changes in the circular-dichroism-measured secondary structure of rhIL-1ra upon PEGylation [43]. Our results show that, HESylation[®] did not alter the secondary structure of anakinra, since the spectra before and after conjugation nearly superimpose.

In another set of experiments, the effect of HESylation[®] on the protein's thermodynamic properties and thermal stability was investigated. A number of studies have shown that glycosylation or PEGylation increases a protein's thermodynamic stability [44-46], such as the work of Rodrigues-Martinez *et al.*, who reported an increase of up to 6 K in the melting temperature (T_m) of α -chymotrypsin upon PEGylation [47]. Similarly, T_m of recombinant human endostatin increased by 15 K upon modification with a single 5 kDa PEG molecule at the N-terminus [48]. Meanwhile, others, such as Gonnelli *et al.* [49] and Plesner *et al.* [50], reported a reduction of T_m after polymer conjugation. The microcalorimetric measurements of HESylated anakinra showed a 4.8 K increase in T_m and a 65% increase in the unfolding enthalpy as well as refolding upon cooling (which was missing in the native protein due to aggregation). These improved thermodynamic properties showed a positive effect on the protein stability under thermal stress, where the conjugated protein did not aggregate for more than 8 h, while the native protein started aggregation already after 30 min. Similar correlation between increased T_m and higher stability was reported in previous studies [45].

The *in vitro* binding affinity was also investigated as an important surrogate for the influence of HES-conjugation on the protein activity. Polymer conjugation to proteins is known to reduce the protein activity due to steric hindrance of the interaction of the protein and the binding receptor, with activity dropping to as low as 7% as in the case of PEG-Interferon α -2a [51]. Site-specific conjugation can greatly improve activity and binding affinity, as it can reduce the interference in protein-receptor interaction. In the current study, the binding kinetics of the native and the conjugated anakinra were evaluated by surface plasmon resonance (SPR) measurements as well as microscale thermophoresis (MST). The latter is a novel entirely optical method for the determination of the binding affinity [52], where the migration of biomolecules along a laser-induced microscopic temperature gradient is mainly governed by protein hydration and surface charge [53]. A binding event leads to alterations of the protein hydration shell, and accordingly changes in the thermophoretic motion [54]. It offers a number of advantages, including minimum sample contamination or perturbation of the molecular

structure due to lack of protein-immobilization, as well as the ability to measure over a wide concentration range (nM to mM), requiring very small sample volumes (< 3 μ l) [52, 53].

SPR measurements of anakinra gave a dissociation constant (K_D) of 0.05 nM, which is close to the reported values of 0.12 [55] and 0.4 nM [56]. Meanwhile, it showed that the on-rate of the HESylated protein decreased by an order of magnitude, most probably due to the shielding effect of the polymer, and possibly the lower diffusion coefficient of the larger conjugate [57], while the off-rates for both proteins are similar, indicating that once the ligand-receptor complex is formed, its dissociation is not influenced by the coupled polymer. The dissociation constants of native and modified anakinra were double-checked by MST, which showed higher K_D values for both proteins, but with the same trend, namely, that the HESylated protein had a multiple-fold higher dissociation constant than the native protein. The lower K_D values obtained by SPR have also been observed by Jecklin *et al.* when they compared SPR to isothermal calorimetry (ITC) and nanoelectrospray ionization mass spectrometry (nESI-MS) [58]. They attributed this observation to issues of protein immobilization, where in case of fast association rates, which are highly affected by mass transport, the binding of the ligand to the protein on the sensor chip is faster than the diffusion of the ligand from the bulk solution to the immobilized protein at the surface [58]. This problem is not present in MST leading to higher apparent K_D values. In general, one infers from both techniques that the conjugated protein is still highly active, retaining a dissociation constant in the low nM range. It is also worth noting that the minor reduction in binding affinity may not influence the *in vivo* performance [5], as observed by Pearce *et al.* who produced hGH mutants with systematically lower binding affinities down to 500-fold [59], and found out that the cellular proliferation of the different mutants was unaffected until the affinity was reduced by more than 30-fold compared to the wild-type protein [59].

Finally, the pharmacokinetic results of both native and HESylated anakinra in male Wistar rats show that HESylation[®] significantly increased the half-life of the protein by 6.5 times, as well as the AUC by 45 times. To compare these results with published information about PEGylated anakinra, quite scarce data can be found in the literature. For instance, a patent by Hakimi *et al.* describes the serum concentrations of native and PEGylated anakinra (conjugate molar mass is 33 kDa, 46% protein content) for up to 24 h after subcutaneous injection into C57BF/6 mice [60]. Analyzing their data using the noncompartmental analysis of PKSolver 2.0, native anakinra shows a $t_{1/2}$ of 1.04 h (which is close to our estimate) while PEGylated anakinra shows a $t_{1/2}$ of 3.53 h. While it is difficult to directly compare the results of HESylated anakinra to those of the published PEGylated one due to differences in the animal

species, molar mass of the polymer and injection route, the results generally show the potential of HESylation[®] for extending the circulation time of proteins.

2.5. Conclusions

The present study reports on the use of HES-conjugation for half-life extension of a model protein, namely rhIL1ra (anakinra). The HESylated protein was synthesized using reductive amination under mild acidic conditions, and purified by anion exchange chromatography, giving a monoHESylated protein with a very good yield. Characterization of the HESylated anakinra by SEC-MALLS and DLS identified the molar mass and size of the protein, respectively, while FTIR showed that HESylation[®] did not alter the protein's secondary structure. Additionally, microcalorimetry showed that HESylation[®] increased the proteins melting point T_m by 4.5 K, as well as the melting enthalpy and refolding upon cooling, which translated into higher stability towards aggregation at 40°C. Meanwhile, HESylated anakinra maintained its high receptor binding affinity in the low nanomolar range as confirmed by SPR and MST. Finally, the pharmacokinetic study of native and HESylated anakinra in Wistar rats showed a marked increase in the protein's half-life and AUC. This study shows that, while HESylation[®] significantly improves the pharmacokinetic parameters of the model protein, anakinra, it does not negatively affect its structure or binding affinity, and increases its stability, making HESylation[®] an attractive HLE technology.

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Chapter III – Highly concentrated formulations

3. Head to Head Comparison of the Formulation and Stability of Concentrated Solutions of HESylated versus PEGylated Anakinra

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Abstract

Although PEGylation of biologics is currently the gold standard for half-life extension, the technology has a number of limitations, most importantly the non-biodegradability of PEG and the extremely high viscosity at high concentrations. HESylation is a promising alternative based on coupling to the biodegradable polymer hydroxyethyl starch (HES). In this study, we are comparing HESylation with PEGylation regarding the effect on the proteins physicochemical properties, as well as on formulation at high concentrations, where protein stability and viscosity can be compromised. For this purpose, the model protein anakinra is coupled to HES or PEG by reductive amination. Results show that coupling of HES or PEG had practically no effect on the protein's secondary structure, and that it reduced protein affinity by one order of magnitude, with HESylated anakinra more affine than the PEGylated protein. The viscosity of HESylated anakinra at protein concentrations up to 75 mg/mL was approximately 40% lower than that of PEG-anakinra. Both conjugates increased the apparent melting temperature of anakinra in concentrated solutions. Finally, HESylated anakinra was superior to PEG-anakinra regarding monomer recovery after 8 weeks of storage at 40°C. These results show that HESylating anakinra offers formulation advantages compared with PEGylation, especially for concentrated protein solutions.

Keywords

PEGylation; HESylation; anakinra; viscosity; protein aggregation; surface plasmon resonance; FTIR; calorimetry; biopharmaceutical characterization

3.1. Introduction

There is currently a large growth in the development of therapeutic peptides and proteins, with many academic institutions and industrial settings introducing engineered proteins having new designs and functionalities [1,2]. However, for many biopharmaceuticals with a molecular weight lower than 60 kDa, such as some cytokines, antibody fragments, or protein scaffolds, the rapid renal filtration and thus short half-life represents a hindrance for successful commercialization due to the need for frequent administration and thereby reduced compliance. Accordingly, these molecules require half-life extension (HLE) technologies to ensure their competitiveness [3]. Additionally, many of them need to be formulated as highly concentrated solutions to enable self-administration of the required high doses by the subcutaneous route [4]. With more than 10 approved products on the market, PEGylation currently represents the gold standard HLE technology [5]. The coupling of polyethylene glycol to peptides and proteins was first introduced in the 1970s by Abuchowski *et al.* [6,7] and later found its way to the market, not only because of extending the circulation time, but also because of imparting many positive characteristics to molecules, such as increased solubility and stability as well as reduced immunogenicity and susceptibility to proteolytic degradation [8]. However, PEGylation suffers from a number of shortcomings, most importantly the fact that PEG is not biodegradable, leading to toxicity concerns, such as vacuolation of the kidney or liver [9–11]. Another safety concern involves the presence of pre-existing or newly developed anti-PEG antibodies, which can limit the therapeutic efficacy of PEGylated proteins [12]. Additionally, highly concentrated solutions of PEGylated proteins can have extremely high viscosities, leading to challenges in production and administration [13,14]. These issues led to the pursuit of better alternatives for PEGylation, with many approaches already commercially under development [15,16]. These alternative technologies can be classified under two main strategies. The first involves the same basic principle employed by PEGylation, that is, increasing the hydrodynamic size of the biopharmaceutical above the renal glomerular filtration threshold. This can be achieved by coupling to biodegradable polymers (such as polysialic acid [17,18]) or fusion to non-folding polypeptides (such as Xten technology, [19,20] or PASylation [21]). The second approach utilizes a binding mechanism, such as binding to the Fc neonatal receptor (FcRn) to make use of the FcRn natural recycling machinery [15]. This can be achieved by fusion to the Fc part of antibodies, or to albumin [15]. HESylation[®] is a promising HLE technology that utilizes the first strategy [22]. It involves protein coupling to the biodegradable and biocompatible semi synthetic polymer hydroxyethyl starch (HES).

HES is widely used as a plasma volume expander for decades, with patients tolerating doses of more than 100 g of HES per day [23]. Such favorable properties have prompted its investigation for biomedical applications [24, 25] and more importantly for polymer–protein conjugation as a promising substitute for PEG [26].

The protein molecule used in this study is anakinra (recombinant human interleukin 1 receptor antagonist, rhIL1-ra). Anakinra is a 17 kDa protein that binds to the IL1 receptor, blocking the inflammatory action of IL1 α and β , and is thus approved for adult patients with rheumatic arthritis who show an inadequate response to other disease-modifying antirheumatic drugs [27]. Because of its short half-life, anakinra has to be administrated by a daily injection of 100 mg, making it an ideal candidate for HLE. We have recently shown that HESylation of anakinra maintains the binding affinity of the protein, has no effect on its secondary structure or stability, and more importantly, increases the elimination half-life more than fivefold [28]. The aim of this study is to use anakinra as a model protein for comparing HESylation versus PEGylation regarding their effect on the physicochemical characteristics of the protein, as well as its formulation and stability, particularly at the challenging situation of high protein concentrations.

3.2. Materials and Methods

3.2.1. Materials

Kineret[®] was purchased from SOBI (Stockholm, Sweden). Branched PEG propionaldehyde (molecular weight (MW) = 40 kDa) was purchased from Jenkem Technologies (Allen, Texas). Activated HES (with a weight average Mw of approximately 85 kDa, number average MW (Mn) of approximately 65 kDa, and a polydispersity of 1.3) was from Fresenius Kabi Deutschland GmbH (Bad Homburg, Germany). All chemicals were used in analytical grade and purchased from VWR (Darmstadt, Germany).

3.2.2. Synthesis and Purification of the Protein Conjugates

3.2.2.1. PEGylation

Anakinra was conjugated to branched PEG propionaldehyde (MW = 40 kDa) on the N-terminus by reductive amination in acetate buffer ($c_{\text{Protein}} = 8 \text{ mg/mL}$, $c_{\text{Buffer}} = 100 \text{ mM}$, pH =

5.0) in a 2.5 molar excess of the polymer using 20 mM sodium cyanoborohydride as the reducing agent under gentle stirring for 16 h at 4°C. The reaction batch was rebuffered for 4 h by a dialysis membrane (Cellu Sep, MW cutoff (MWCO) 3.5 kDa; Scienova GmbH, Jena, Germany) against 5 L of a 20 mM TRIS buffer pH = 8.0 at 4°C before loading on a self-packed XK 50/20 filled with Q Sepharose HP (GE Healthcare, Uppsala, Sweden) for anion-exchange chromatography at a flow rate of 0.5 mL/min. The monoPEGylated protein was separated from the multi-conjugates and the unreacted protein using 10 column volumes in a linear salt gradient using Tris buffer with 250 mM NaCl on an ÄKTA Purifier (GE Healthcare) at a flow rate of 3 mL/min. For further experiments, the pooled fractions of mono-conjugate were rebuffered against citrate-saline- EDTA (CSE) buffer ($c_{\text{Citrate}} = 10 \text{ mM}$, $c_{\text{NaCl}} = 140 \text{ mM}$, $c_{\text{EDTA}} = 0.5 \text{ mM}$, pH = 6.5) by tangential flow filtration (TFF) using a MinimateTM capsule with OmegaTM 10 kDa membrane (PALL Scientific, East Hills, New York). The concentration was measured by UV-spectroscopy at 280 nm with an extinction coefficient of $13,392 \text{ M}^{-1} \text{ cm}^{-1}$ [29].

3.2.2.2. HESylation

Anakinra was conjugated to HES propionaldehyde targeting protein's N- terminus by reductive amination in acetate buffer. In a 1 L glass reaction vessel equipped with a thermo jacket and a blade stirrer, 2.5 g protein (19 mL; 134 mg/mL) were diluted into 220 mL 0.1 M sodium acetate buffer (pH = 5.0) prechilled to 5°C. Hydroxyethyl starch (Mn ~ 68 kDa) carrying a reactive propionaldehyde group (4-amino- N-(3-oxopropyl)butanamide [30]) was dissolved in the same buffer to yield a 40% (w/v) solution and 75 mL of the HES solution (representing a 3:1 molar ratio of polymer to protein) were combined with the protein under moderate stirring. The reductive amination reaction was started by addition of 12.5 mL of a freshly prepared 0.5 M NaCNBH₃ solution in water and incubated overnight (18 h) under moderate stirring at 5°C. The conjugate was purified by anion-exchange chromatography using Q Sepharose HP material and an Äkta Purifier 100 chromatography system (both GE Healthcare, Munich, Germany). The system was operated at room temperature with a typical flow rate of 10 mL/min. The column (XK 26/20) was first equilibrated with 5 CV of eluent A (10 mM Tris acetate, pH = 8.0). The reductive amination reaction mixture was 10-fold diluted with eluent A, the pH adjusted to pH 8.0 using NH₄OH solution and beta-mercaptoethanol added to a final concentration of 1 mM to destroy potential disulfide-mediated protein dimers. Up to 800 mg of conjugate was then loaded onto the column at a flow rate of 10 mL/min, followed by a washing step with 2 CV 5% eluent B (eluent A + 250 mM NaCl) to remove the unbound,

excessive HES polymer. The conjugate was eluted in a step gradient at 25% eluent B for 4 CV. Peak fractions were collected and subsequently a buffer exchange to CSE (10 mM citrate, 140 mM sodium chloride, 0.5 mM EDTA, pH = 6.5) buffer w/o Tween 80 and up-concentration performed by TFF using a 50 cm² PES (polyethersulfone) membrane capsule with a MWCO of 10 kDa operated on a Minimate™ benchtop system (both from Pall, Dreieich, Germany). The retentate of the TFF step was subjected to a 0.22 µm filtration step, aliquots of the filtered solution shock-frozen in liquid nitrogen and stored at –80°C until further use.

3.2.3. SEC–MALLS Measurement for the Determination of the Molecular Weight and Size of the Conjugates

The MW of native, PEGylated, and HESylated anakinra were investigated by size-exclusion chromatography–multi- angle laser light scattering (SEC–MALLS) on the AF2000 Focus (Postnova Analytics, Landsberg/Lech, Germany) equipped with a MALLS miniDAWN Tristar detector (Wyatt Technology, Dernbach, Germany), refractive index detector (PN 3150; Postnova Analytics) and a Shimadzu SPD-10A UV-VIS Detector (Shimadzu, Duisburg, Germany). For SEC separation, a Superose 6 10/300 GL column (GE Healthcare) was used with CSE buffer as the running phase including 0.02% sodium azide at a flow rate of 0.5 mL/min over 45 min. Each run was performed by the injection of 50 µg, based on the protein weight. The data were analyzed by the Astra software version 5.0 (Wyatt Technology). The protein conjugate analyses were performed by the Wyatt protein conjugate application embedded in the Astra 5.0 software. UV extinction coefficient for Anakinra was 13,392 M⁻¹ cm⁻¹ [29]; dn/dc for anakinra was 0.1850 mL/g, for PEG 0.1340 mL/g, [31] and HES 0.1460 mL/g [32].

3.2.4. Hydrodynamic Radius

The hydrodynamic radius was measured by dynamic light scattering (DLS) using the Malvern Zetasizer (Malvern Instruments, Herrenberg, Germany). Native, PEGylated, and HESylated anakinra were diluted to a final concentration of 1 mg/mL (based on the protein). Before measurement, all samples were filtered through a 0.2 µm filter. The hydrodynamic radius is

expressed as the Z- average, together with the polydispersity index as an indication for the size distribution.

3.2.5. FTIR

FTIR spectroscopy experiments were performed using the Bruker Tensor 27 FTIR with the Bruker AquaSpec Cell (Bruker Optics, Ettlingen, Germany). The sensor was cooled with liquid nitrogen and a constant gaseous nitrogen flow. The samples were analyzed at a concentration of 3 mg/mL (based on the protein part) in 240 scans against CSE buffer. Using the OPUS Software, the second derivative of the spectrum for each sample was obtained in the range of 1600 to 1700 cm^{-1} . The curves were normalized by vector normalization.

3.2.6. Biacore Analysis for *in vitro* Binding Affinity

The binding affinity was analyzed by surface plasmon resonance (SPR) using a Biacore T100 instrument (Biacore AB, Uppsala, Sweden). The recombinant IL-1 receptor type I (R&D Systems, Wiesbaden, Germany) was immobilized by standard EDC/NHS coupling on a CM3 chip in acetate buffer ($c = 20 \text{ mM}$, $\text{pH} = 4.5$) to a final response of $\approx 570 \text{ RU}$. A kinetic study was performed for native, PEG- and HESylated anakinra by choosing 180 s for the on-rate and 300 s for the off-rate with a constant flow rate of 30 $\mu\text{L}/\text{min}$ measured at room temperature. After the 300 s dissociation of the analytes, the receptor was regenerated with Glycine 5 mM $\text{pH} = 2.0$ for 6 s. Different sample concentrations were diluted in HBS-EP running buffer (contains HEPES 10 mM, sodium chloride 150 mM, EDTA 3 mM and P-20 0.005%). The data were subtracted from an uncoated cell and normalized to their baseline using the BIAcore T200 Evaluation Software. The overlaid curves showed the loss in activity by a decreased response at different concentrations.

3.2.7. Preparation of Highly Concentrated Solutions

Up-concentration of the protein solutions was performed by ultrafiltration in Vivaspin 20 centrifugators using a MWCO of 10 kDa for the conjugates and 3.5 kDa for the unmodified protein. The tubes were placed into a Sigma 4K15 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) and rotated at 10,000 rpm ($= 14,243 \text{ g}$) for 3– 4 h at 4°C. The

protein concentration was verified by UV measurement (NanoDrop; Thermo Scientific, Schwerte, Germany) using the aforementioned extinction coefficient of the protein. For further experiments, the samples were diluted in the formulation buffer to the desired protein concentration.

3.2.8. Differential Scanning Calorimetry

Thermal properties of the highly concentrated protein solutions were investigated by differential scanning calorimetry on the Netzsch DSC (Netzsch- Gerätebau, Selb, Germany). Therefore, 100 μL of native, PEGylated, and HESylated anakinra with a sample concentration of 25, 50, and 75 mg/mL (relabased on the protein) was filled in 100 μL aluminum crucibles, hermetically sealed, and measured against CSE buffer. The folding process was followed by a heating gradient from 20°C to 95°C with a heating rate of 1°C/ min. Data analysis was determined by the Proteus software. The curves were smoothed by the program to determine the apparent T_m as the peak maximum of the melting process. Additionally, the temperature describing the onset of unfolding, T_{onset} , was determined by integrating the curve in Origin software and fitting the curve using a Boltzmann equation as described by Menzen and Friess [33].

3.2.9. Viscosity

The rheological properties as a function of protein concentration of the wild type and both conjugates were investigated on the mVROC device (Rheosense Inc., San Ramon, CA, USA) at 20°C. All samples were diluted in CSE buffer to 25, 50, and 75 mg/mL and analyzed at a shear rate of 314 s^{-1} . The viscosity of Cimzia® (UCB, Brussels, Belgium) measured under equivalent conditions served as a benchmark. The mentioned setup was also used to investigate the influence of pH as well as sodium chloride on solution viscosity.

3.2.10. Storage Stability: SEC

The formation of soluble aggregates and the levels of monomeric protein were determined by SE-HPLC measurements on a Spectra-Physics System P2000 (ThermoFisher Scientific, Henningsdorf, Germany) equipped with a TSKgel G3000 SWxl (TOSOH Bioscience GmbH, Stuttgart, Germany) with CSE buffer as the running solvent at a flow rate of 0.5 mL/min over

35 min. The peak areas of the chromatograms were used to quantify the amount of soluble aggregates and the monomeric fraction of each sample. Protein standards with a known concentration were stored at 2– 8°C and served as controls for the thermal incubation experiment. All samples were diluted to 1 mg/mL and centrifuged to analyze the supernatant. Each SE-HPLC run was performed by the injection of 50 µg of protein, detection wavelength was 280 nm.

3.3. Results

3.3.1. Synthesis and Purification

In case of both PEGylation and HESylation, the coupling efficiency was similar (approximately 75%). After purification using anion exchange chromatography, mono conjugated protein could be obtained, with a purity > 98% for both HESylated and PEGylated anakinra as determined by SEC and RP-HPLC, with a cumulated process yield of 65% – 70%.

3.3.2. Determination of the Molecular Weight and Hydrodynamic Size

Size-exclusion chromatography combined with MALLS, UV, and RI detectors was used for the calculation of the MW as well as quantification of the extent of coupling and/or aggregation of the conjugated proteins. The MW of native anakinra was found to be 16.6 kDa, in good agreement with the theoretical value of 17.26 kDa (**Fig. 1a and Table 1**). PEGylated and HESylated anakinra showed a MW of 53.5 and 105.5 kDa, respectively (**Figs. 1b and 1c and Table 1**), which is also quite close to the hypothetical masses of 57 and 102 kDa for mono conjugated proteins. In case of PEGylated anakinra, a small peak (peak area < 0.3%) of higher MW species can be observed before the monomer peak. It belongs to a dimer of mono-PEGylated protein (and not to a diPEGylated one) as confirmed by the doubling of the MW of the monoconjugate to 105.6 kDa. Regarding MW distribution, HESylated anakinra showed a broader distribution compared with the PEGylated protein (PDI HES-anakinra = 1.244 vs. PDI PEG-anakinra = 1.004), which is due to the natural origin of HES.

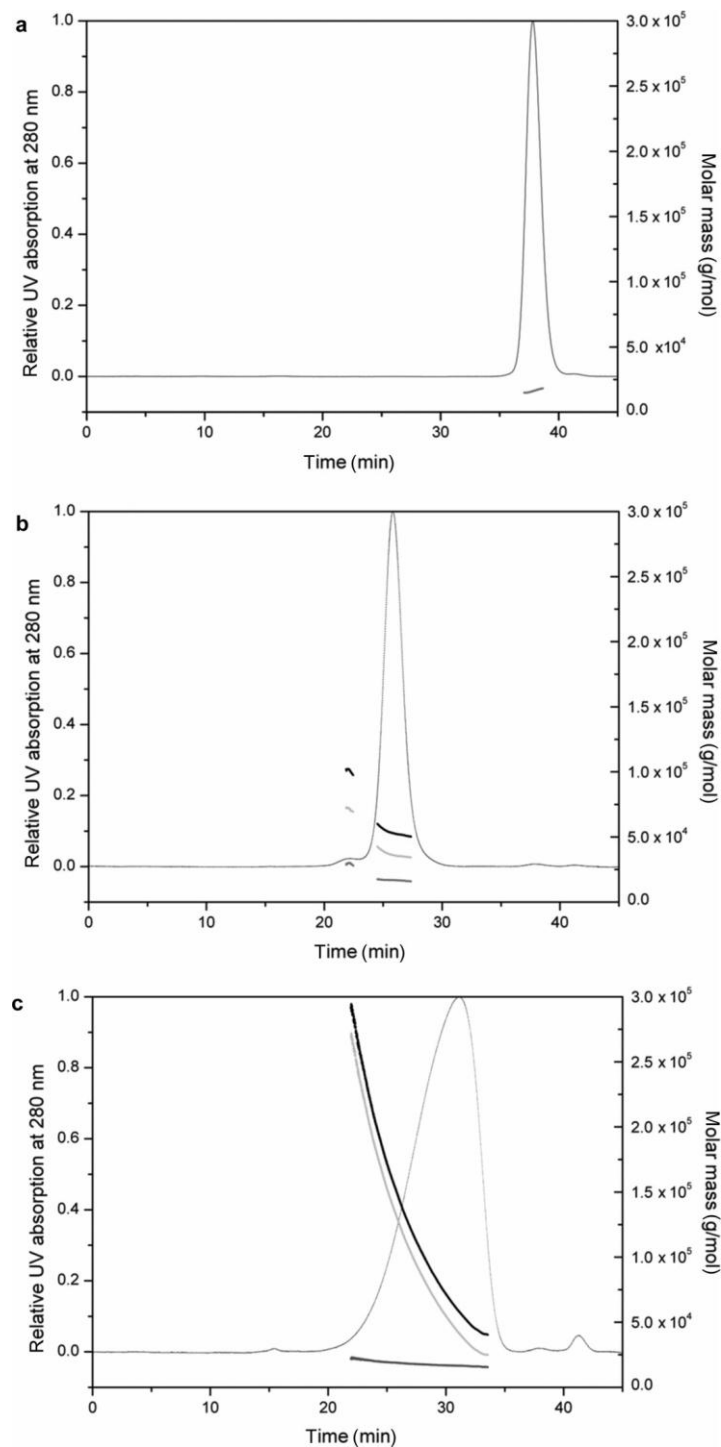


Figure 1: SEC–MALLS experiment for determining the molar mass of (a) native, (b) PEGylated, and (c) HESylated anakinra. The molar mass distributions of the conjugate, the protein part, and the polymer part are expressed in black, dark gray, and light gray lines, respectively.

Dynamic light scattering was used to determine the hydrodynamic size of the three proteins. Results in **Table 1** show that the diameter of anakinra is very close to that reported earlier

[34]. Meanwhile, polymer conjugation to PEG or HES increases the size by threefold to approximately 14.5 nm, with no significant difference between the two polymers used.

Sample	M _w [kDa]	Polydispersity	Hydrodynamic diameter [nm]	PDI
Native anakinra	16.6	1.000	4.36 ± 0.18	0.16 ± 0.03
PEGylated anakinra	53.5	1.004	14.40 ± 0.27	0.08 ± 0.02
HESylated anakinra	105.5	1.244	14.73 ± 0.25	0.20 ± 0.01

Table 1: Weight Average Molecular Weight (M_w) and Polydispersity as determined by SEC MALLS measurements, as well hydrodynamic diameter and Polydispersity Index as determined by DLS Analysis for the native, PEGylated, and HESylated anakinra

3.3.3. FTIR Analyses for Changes in Secondary Structure

The second derivative spectrum of anakinra in **Figure 2** shows the main predominant beta sheet conformation (peak at 1640 cm⁻¹) and beta-turn structures (peak at 1685 cm⁻¹) [35]. The spectrum of HESylated anakinra correlates well with that of the native protein, whereas PEGylated anakinra shows a minor shift in the beta sheet peak from 1640 to 1641 cm⁻¹. Therefore, it can be assumed that neither PEGylation nor HESylation under the chosen reaction and purification conditions led to significant changes in the secondary structure of the protein.

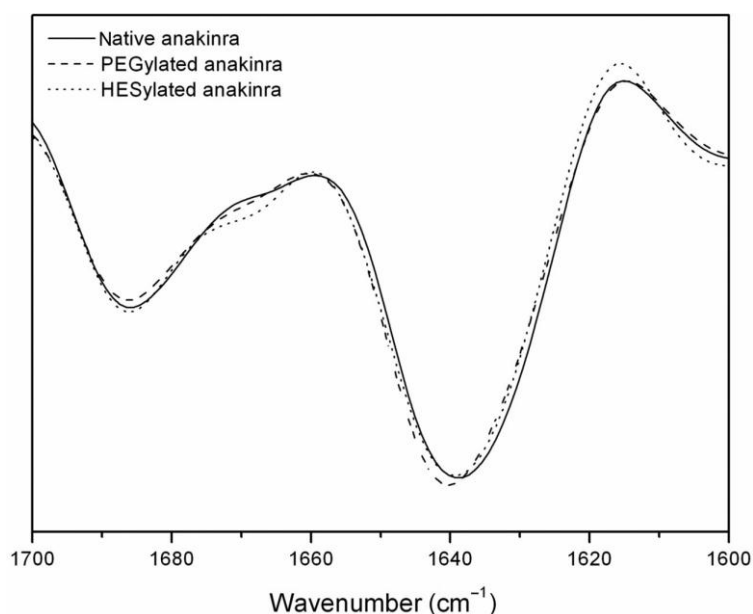


Figure 2: Second- derivative amide I FTIR spectra of native anakinra (solid line), PEGylated anakinra (dashed line), and HESylated anakinra (dotted line).

3.3.4. *In Vitro* Binding Affinity

To investigate the effect of PEGylation and HESylation on the apparent binding affinity, the binding kinetics of the native and conjugated anakinra was evaluated by SPR. Recombinant IL-1 receptor type I was immobilized on a CM3 chip with a low density to minimize mass transport and rebinding. **Table 2** demonstrates the on- and off-rates, as well as the k_D value of native, PEGylated, and HESylated anakinra. The wild type protein showed the fastest on-rate followed by a very slow off- rate (**Fig. 3 and Table 2**), which led to a k_D value of 0.05 nM. The on-rates of the PEGylated and HESylated anakinra are nearly one order of magnitude lower than the native protein, whereas the off-rates for all three proteins are similar, indicating that dissociation of the ligand-receptor complex is not influenced by the coupled polymer. This leads to higher k_D values for the protein conjugates relative to the native protein ($k_{D_{\text{PEG-anakinra}}} = 0.81 \text{ nM}$, $k_{D_{\text{HES-anakinra}}} = 0.32 \text{ nM}$).

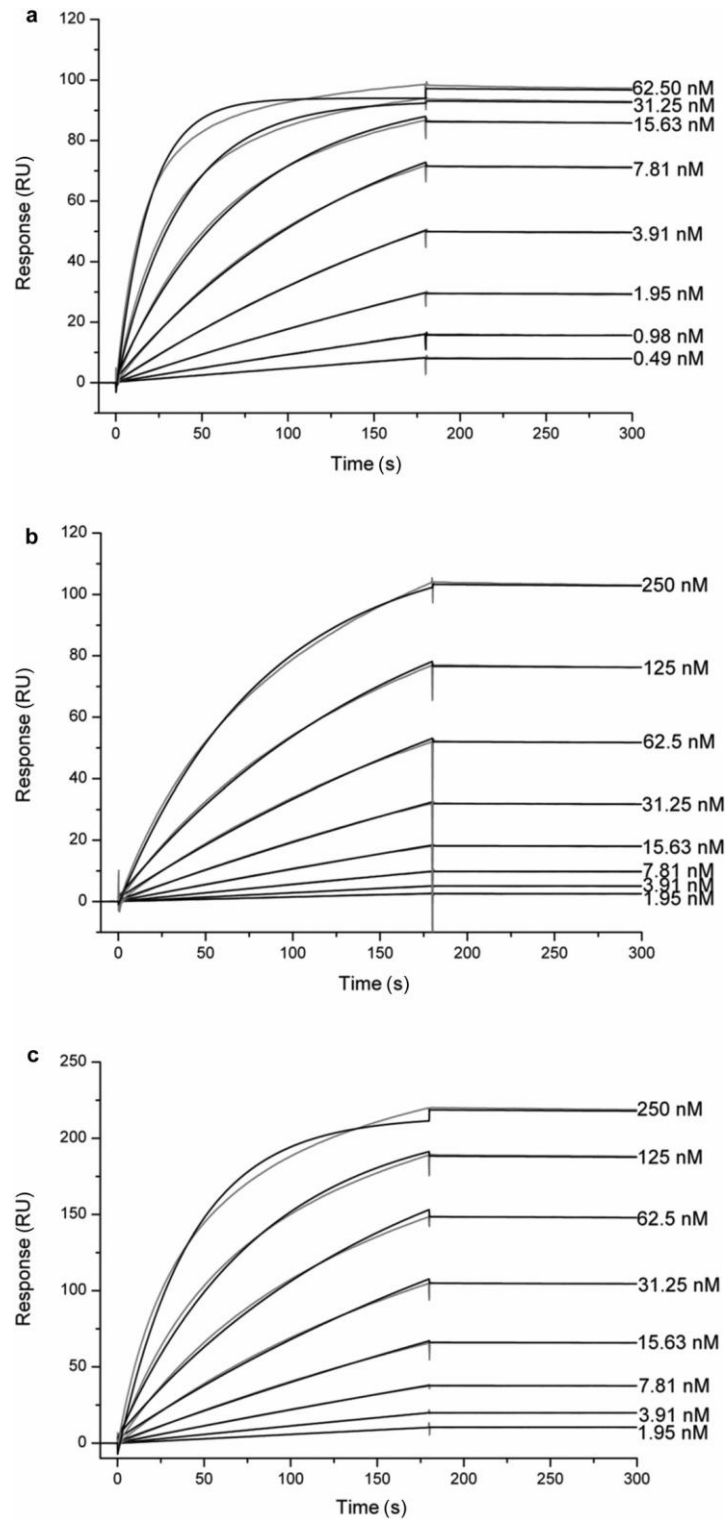


Figure 3: SPR analyses for the binding of (a) native anakinra, (b) PEGylated anakinra and (c) HESylated anakinra to IL-1 receptor type I. All binding curves (dark gray lines) were fitted by Langmuir (1:1) binding isotherm (black lines).

SPR			
Sample	k_{on}	k_{off}	k_D
	[1/Ms]	[1/s]	[nM]
Native anakinra	8.54E+5	3.91E-5	0.05
PEGylated anakinra	4.75E+4	3.87E-5	0.81
HESylated anakinra	9.61E+4	3.11E-5	0.32

Table 2: On-rates, off-rates and dissociation constants for native, PEGylated and HESylated anakinra from SPR measurements.

3.3.5. Properties of Highly Concentrated Solutions

3.3.5.1. Viscosity Measurement

In the current study, we measured the viscosity of all three proteins using the microchip technology of the mVROC system [36]. Results show that increasing the concentration of the native protein from 25 to 75 mg/mL (1.45– 4.35 mM) has little effect on viscosity. Meanwhile, the viscosity of the PEGylated protein solution shows a dramatic increase of one order of magnitude over the same molar concentration range (see **Fig. 4**). In comparison, the viscosity of the HESylated protein is only approximately 60% of the PEGylated protein at the same molar concentrations. Additionally, we studied the effect of pH and ionic strength on the viscosity of the conjugates. Results show that the viscosity of anakinra did not change in slightly alkaline conditions, but nearly doubled at pH 4 (**Fig. 5, left**). The same trend was seen with anakinra conjugates, which showed much higher viscosity in acidic pH compared with pH = 6.5 or 8. The viscosity of all three proteins did not show any significant changes over the studied salt concentration range (**Fig. 5, right**).

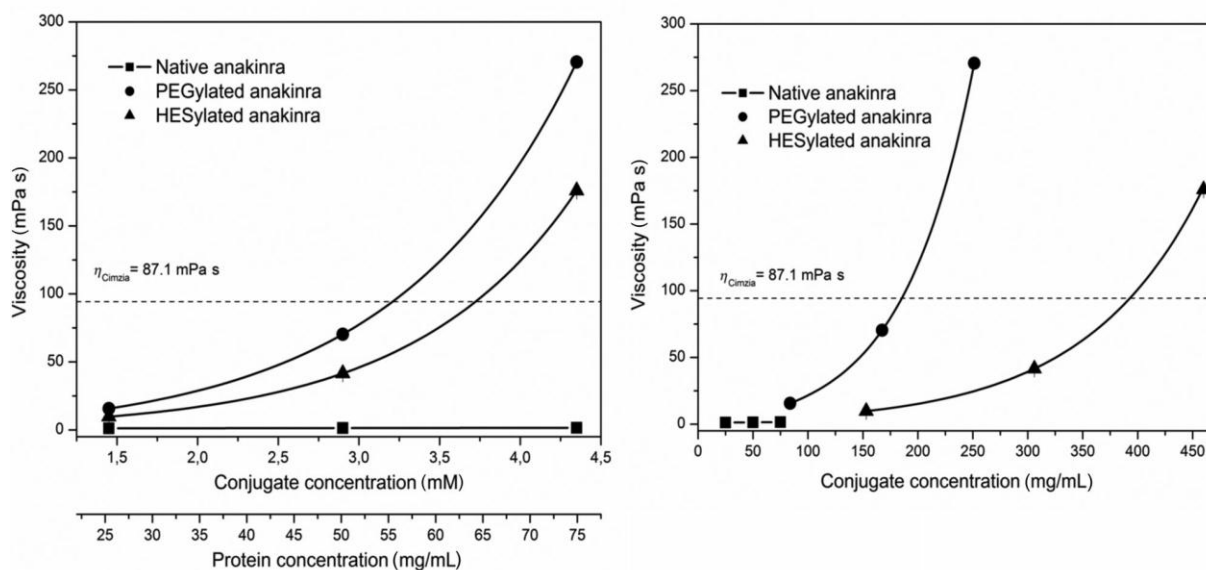


Figure 4: Viscosity of anakinra, PEGylated-, and HESylated anakinra as a function of conjugate concentration. Left: The x-axis represents the conjugate concentration in mM or the concentration of the protein part in mg/mL. Right: the x-axis represents the conjugate concentration in mg/mL. The lines are fitted by exponential function. The horizontal dashed line represents the viscosity of Cimzia[®] (conjugate concentration of 200 mg/mL), which is included as a benchmark.

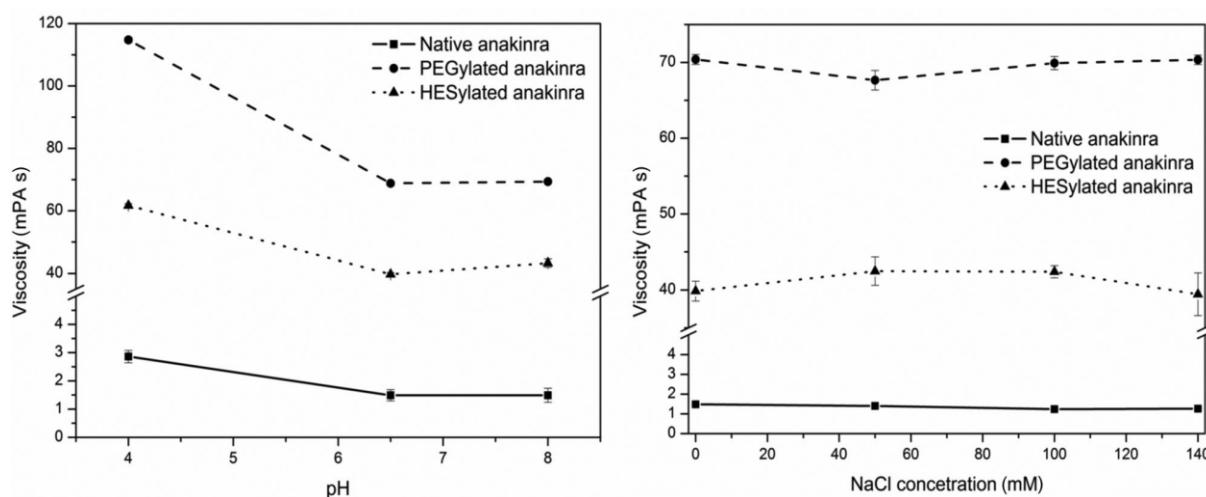


Figure 5: Viscosity of anakinra, PEGylated, and HESylated anakinra as a function of pH (left) and NaCl concentration (right). Protein concentration was 50 mg/mL.

3.3.5.2. Thermal Analysis

The thermal properties of different concentrations of native and conjugated anakinra were evaluated by DSC measurements of the different proteins at 25, 50, and 75 mg/mL protein concentration. Anakinra is known to precipitate upon unfolding in thermal analysis experiments, [37] and we had the same observation during earlier experiments [28]. Accordingly, a robust determination of the protein melting temperature (T_m) was not possible, and the peak maximum was regarded as apparent T_m . Additionally, we identified the temperature corresponding to the onset of protein unfolding T_{onset} . The results in **Figure 6** show that the apparent T_m of anakinra decreases slightly with increasing concentration over the studied range. Meanwhile, both protein conjugates show a much higher apparent T_m compared with native anakinra over the studied concentration range, and show the same pattern of decreasing apparent T_m with increasing concentration, except for HESylated anakinra, which shows a slight increase in apparent T_m at the highest concentration tested (75 mg/mL protein concentration). It is worth noting that PEGylated anakinra shows a slightly higher apparent T_m compared with HESylated anakinra. As for T_{onset} , the values for native anakinra show a slight decrease with increasing concentration, although the differences are not significant because of the large scatter in values. Meanwhile, HESylated and PEGylated proteins have a much higher T_{onset} compared with native anakinra, with a clear trend towards lower temperature with increasing concentration. There is no difference HESylated and PEGylated anakinra at 25 mg/mL, but at higher concentrations, T_{onset} of PEGylated anakinra is higher, although with large scatter in values.

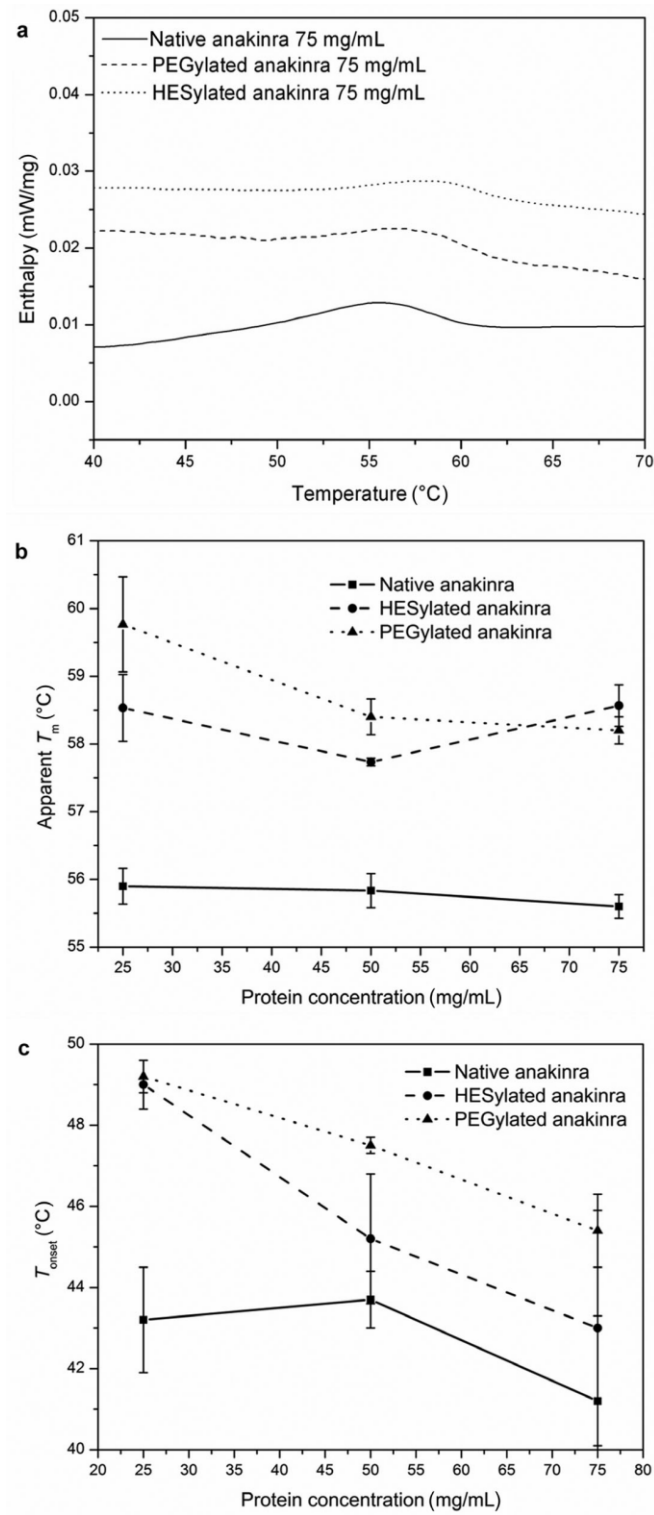


Figure 6: DSC thermogram for native, HESylated, and PEGylated anakinra at a protein concentration of 75 mg/mL (a), the apparent melting temperature (T_m) of all three proteins as a function of protein concentration (b), as well as the temperature corresponding to the onset of unfolding (T_{onset}) as a function of protein concentration (c).

3.3.5.3. Thermal Stability upon Storage at 40°C

The thermal stability of anakinra and its conjugates was evaluated by storing them at three different protein concentrations (25, 50, and 75 mg/mL) at 40°C for 8 weeks, and then evaluated by estimating the monomer recovery by SEC. Results in **Figure 7** show that native anakinra at 75 mg/mL degrades rapidly, so that only 63.9% of the monomer is recovered after 8 weeks. In contrast, PEGylation leads to an improved protein stability and reduction in protein loss, with a recovery of 71.9% at the end of the study for the 75 mg/mL concentration. HESylation on the other hand results in a surprisingly high protein recovery of 88.2% monomer at the same concentration and a lower degree of higher aggregates formation compared with PEGylation (see Fig. 7).

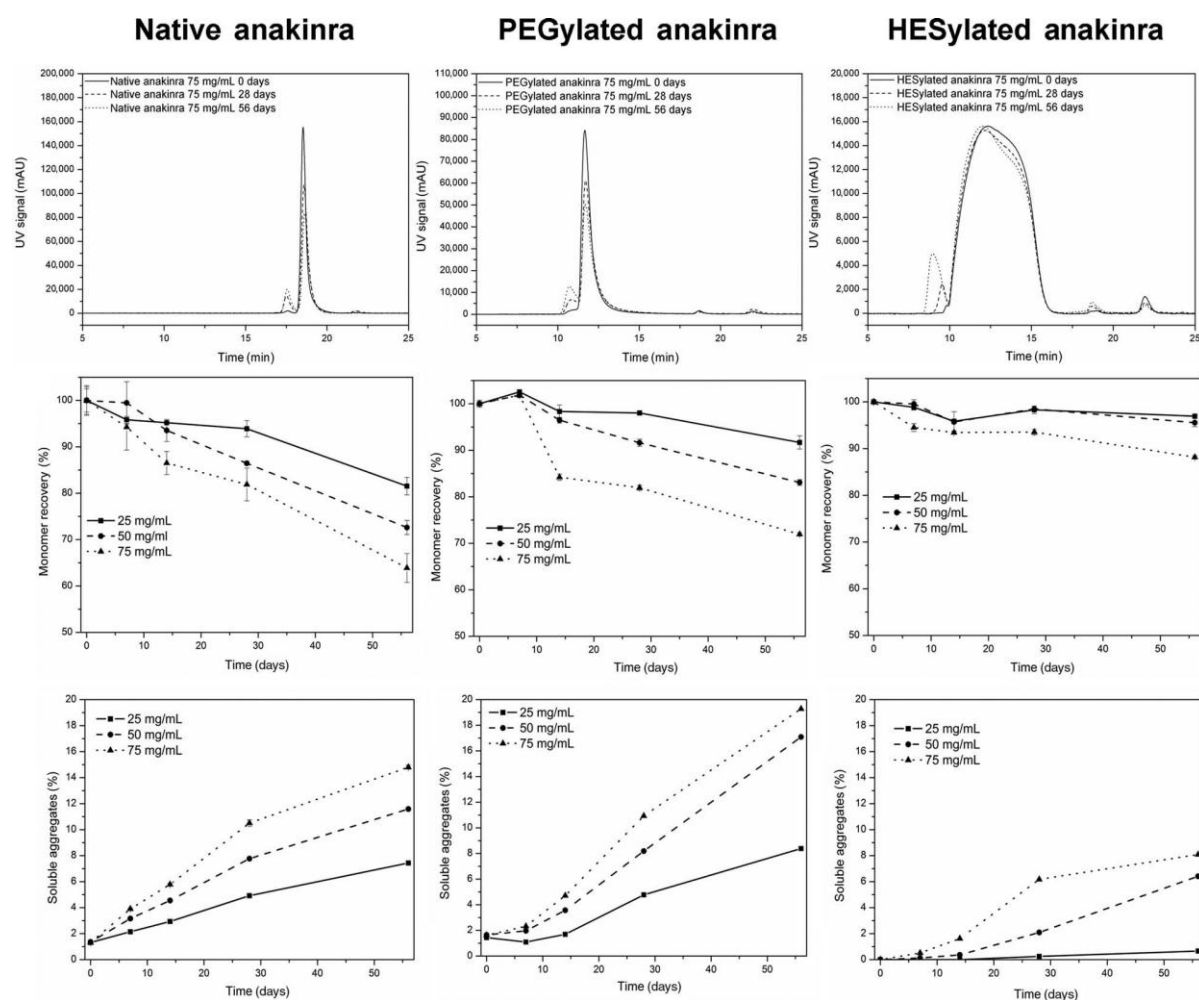


Figure 7: SEC chromatograms for native, PEGylated, and HESylated anakinra samples at three different protein concentrations (25, 50, and 75 mg/mL) stored at 40°C for 8 weeks (Upper panel). The percentage of monomer recovery of the three proteins as a function storage time (middle panel). The percentage of soluble aggregates for the three proteins as a function of storage time (lower panel).

3.4. Discussion

Although PEGylation is currently the benchmark technology in HLE, it suffers from a number of drawbacks, including PEG's non-biodegradability, [38] and the high viscosity of concentrated PEG solutions, complicating processing and injectability [3]. Accordingly, alternative technologies are being pursued, with HESylation being a promising HLE approach [28]. In the current study, we are comparing HESylation with PEGylation regarding the effect on the physicochemical properties of proteins, as well as protein formulation at high concentration. The latter case can be associated with protein stability and viscosity issues, and the aim is to find out whether HESylation could offer advantages over PEGylation in this respect. For this purpose, anakinra was used as a model protein. Anakinra is a small 17 kDa protein that binds to IL-1 receptor with a short terminal half-life of 108 min [27]. As a result, daily administration of 100 mg protein as subcutaneous injection in the form of a highly concentrated solution (150 mg/mL) is required. Additionally, the protein is well studied with many literature reports about its biophysical properties and formulation, [34, 39– 42] thus serving as a useful model protein. There are a few literature reports on the PEGylation of anakinra to increase its circulation time and reduce the dosing frequency [43, 44]. They involved coupling PEG at the N-terminus, [43] as well as at cysteine and lysine residues, [44] although with much smaller PEG molecules compared with the ones used in the current study (5 vs. 40 kDa). The site-specific conjugation at the N-terminus employed in this study using reductive amination has been used in the development of Peg- filgrastim, a commercialized PEGylated form of GCSF [45]. The coupling chemistry is based on the difference in pKa between the ϵ - amino groups of lysine (pKa = 10.1) and that of the N- terminal amino group (pKa = 7.8), leading to a preferred Schiff's base formation (and subsequent reduction) to the N-terminus at slightly acidic conditions [46,47]. This chemical reaction is employed in the current study to couple anakinra to HES at a single terminal aldehyde group, as described earlier [28]. Previous reports on HESylation involved coupling several peptides or small molecules to one HES chain, [26,48,49] as well as site-specific coupling [22] with a defined 1:1 stoichiometry. The physicochemical analysis of the conjugates involved measurements using SEC–MALLS, DLS, and FTIR. SEC– MALLS confirmed the conjugation and the purity of the mono- conjugated proteins, where the MWs of the conjugates were in good agreement with the theoretical values (see **Table 1**). The HESylated protein showed a higher polydispersity compared with the PEGylated one. This is because of the natural origin of the polymer, with polydispersities for commercially available, HES-based plasma volume expanders as high as 4.5 [50]. However,

by polymer fractionation, it is possible to obtain HES fractions with much narrower size distribution as in the case of the starting material used in this study. Although the two conjugates differ considerably in MW (105.5 vs. 53.5 kDa for the HESylated and PEGylated proteins, respectively), their hydrodynamic sizes are very close (see **Table 1**). This similarity in hydrodynamic size despite the difference in MW is attributed to the fact that HES is a rigid branched polymer, [50] whereas PEG is very flexible and less branched (two branches only, each having a MW of 20 kDa). This architecture of HES forces it to attain a smaller size compared with flexible polymers with similar MW. As the main mechanism of HLE of both polymer conjugates is based on the molecule's hydrodynamic size (irrespective of the MW), these two conjugates with similar sizes were chosen for the current study. FTIR analysis of anakinra was employed frequently to identify changes in protein structure, such as structural changes due to dimerization or aggregate formation in liquid form, [35,39] or due to interaction with excipients [37].

Accordingly, it was employed in this study to evaluate the effect of polymer conjugation on the protein's secondary structure. In this respect, the majority of literature reports support the opinion that conjugation has no effect on a protein's secondary or tertiary structure [51,52]. However, a few studies describe changes in protein conformation upon PEGylation [8,53]. For instance, Yu *et al.* reported changes in the secondary structure of anakinra upon PEGylation. Our results show that, contrary to the aforementioned study, neither PEGylation nor HESylation led to significant changes in anakinra's secondary structure, which is in accordance with the conventional notion in this respect [51,52]. Surface plasmon resonance analysis was used to evaluate the binding affinity of the conjugated proteins. The kD value of native anakinra is quite close to the reported values of 0.12 [55] or 0.4 nM [56] (see **Table 2**). The on-rates of the PEGylated and HESylated anakinra are nearly one order of magnitude lower than the native protein, with the on-rate of PEGylated anakinra nearly half that of the HESylated one (see **Table 2**). This reduction is probably because of the shielding effect of the polymers [57]. The off-rates are, however, similar for all three proteins, indicating that the dissociation of the ligand–receptor complex is not influenced by the coupled polymer. This leads to higher kD values for the protein conjugates relative to the native protein. These results show that both conjugates remain highly active, with the HESylated protein being more affine than the PEGylated one.

In the current study, the formulation of highly concentrated solutions of protein conjugates was evaluated. Generally, the development of highly concentrated protein solutions is associ-

ated with many analytical and formulation challenges [58]. This is mainly because of the higher tendency for aggregation upon protein crowding, making it one of the most important protein degradation routes at high concentration [58]. Additionally, proteins are liable to reversibly self-associate in crowded conditions, leading to the increase in viscosity. This is aggravated in case of polymer–protein conjugates because of the presence of the polymeric component, which tends to entangle at high concentrations, leading to an extreme increase in viscosity. Though the formulation of highly concentrated protein solutions is gaining increasing attention, surprisingly little is known in the literature about the formulation of concentrated solutions of polymer–protein conjugates. In this study, we investigated the viscosity of the highly concentrated conjugates as well as their thermal properties and thermal stability. On an industrial scale, TFF is used for the final polishing and up-concentration of protein solutions. Because of the demanding material consumption of high concentration solutions, and the lack of sufficient protein amounts, we had to use small-scale methods for up-concentration, including either dialysis or ultrafiltration with stirred cells or centrifugal filters. The method employed for up-concentration can have an influence on protein stability as shown by Eppler *et al.*, [59] who demonstrated that the method showing the best comparability with TFF in terms of protein stability was centrifugal filtration [59]. Accordingly, we employed this method in sample preparation. The mVROC instrument was used for viscosity measurements, where it applies for this purpose a microchip-based technology [36]. It was used to measure the viscosity of high concentration antibody solutions, and has the advantage of low sample volume, thus saving material costs [60]. Additionally, it is devoid of measurement artifacts due to surface tension effects as it is the case with the widely used cone and plate rheometers [61]. Results show that the viscosity of PEGylated anakinra drastically increases with increasing protein concentration in comparison with that of native anakinra (see **Fig. 4**). Similar behavior was reported earlier, where the viscosity of solutions of PEG or PEGylated proteins is known to increase exponentially with the concentration [14,62]. For instance, the viscosity of canine haemoglobin modified with up to six molecules of 5 kDa PEG increased “in a slightly exponential fashion” with concentration [14]. Meanwhile, the HESylated protein shows approximately 60% of the viscosity of the PEGylated protein at equivalent molar concentrations, which is clearly a benefit and would translate into a dramatic improvement in the force required for injection. The latter is proportional to viscosity, [63,64] and is important in determining the injectability either by patients or by autoinjectors [63,64]. The observed lower viscosity of HESylated anakinra can be explained by the highly branched and stiff architecture of HES, [50] which makes it closer to a hard sphere and less prone to entanglements

compared with the highly flexible PEG structure. pH and ionic strength are known to influence the viscosity of proteins [58,65,66]. For instance, salts can shield the protein surface charges, and thus prevent attractive electrostatic interactions at high protein concentrations [67]. Similarly, pH changes the protein surface charges, and accordingly the intermolecular interactions [65]. As seen in **Figure 5**, the conjugates followed the same trend as native anakinra, where they showed higher viscosities in acidic pH compared to pH 6.5 or 8, however HESylated anakinra maintained its nearly 40% reduction in viscosity compared with its PEGylated counterpart. Meanwhile, changing the salt concentration had no effect on viscosity of all three proteins, indicating that the attractive electrostatic interactions do not play an important role at pH 6.5, and that the high viscosity of the conjugates is due to the polymer part of the molecule, which is not affected by the employed NaCl concentrations. The thermal properties of the three proteins at high concentrations were evaluated using DSC. The protein melting temperature T_m represents the temperature at which 50% of the protein molecules are folded and 50% are unfolded, and it is a thermodynamic parameter for a reversible process. The peak maximum of the endotherm usually corresponds to T_m , but is amenable to errors if the peak is asymmetric, thus curve analysis using robust models, such as the two-state model could be used to correctly calculate T_m . However, anakinra is known to precipitate during thermal analysis, [28,37] making the process of unfolding irreversible and hindering thermodynamic analysis. Accordingly, the peak maximum was regarded as apparent T_m , and was used for comparative analysis. Additionally, T_{onset} was computed to designate the onset of protein unfolding. The latter is gaining importance as an indicator for protein stability, [68] as it takes into account the width of the endothermic peak. Indeed, two proteins could have the same T_m , but the protein with larger peak width (smaller T_{onset}) is expected to have lower thermal stability [68]. Results show that, for all three proteins, the apparent melting temperature decreases with increasing concentration, a behavior reported earlier for some proteins [69]. Additionally, one notices that conjugation to both polymers leads to increase in both apparent T_m and T_{onset} . This is in agreement with many earlier reports showing that protein PEGylation or glycosylation increase the protein melting point [70–72]. Finally, comparing the apparent T_m for both protein conjugates shows that the PEGylated protein has a higher T_m compared with the HESylated one, indicating a higher thermal stability, however, T_{onset} temperatures show that there is little to no difference between the two conjugates. Finally, protein stability was evaluated by storage at three different concentrations under elevated temperature for 8 weeks. Results in **Figure 7** show a significant decrease in monomer recovery of anakinra with increasing protein concentration. This effect has already been observed for ana-

kinra and other proteins, [39,73] and is explained by increased macromolecular crowding and high total volume occupancy [58,74–77]. Although both protein conjugates show improved monomer recovery, HESylation shows a surprisingly higher monomer recovery compared with PEGylation at all the three studied concentrations. The surprising difference in protein stability might be due to differences in the nature of the polymers, where PEG is known to change the polarity and dielectric constant of aqueous solutions [78]. Another possible reason could be the well-known tendency of PEG to generate peroxides at high temperatures, [79–81] which could have led to the observed increase in protein degradation compared with the HESylated protein.

3.5. Conclusion

The present study reports on the synthesis of PEGylated and HESylated anakinra and the comparison of the two polymer conjugates, regarding protein physicochemical properties and formulation at high protein concentrations. Both conjugates were synthesized using reductive amination under mild acidic conditions to produce predominantly mono-conjugated proteins, as proven by the physicochemical characterization of the conjugates using SEC–MALLS experiments. DLS measurements showed that both conjugates have similar hydrodynamic size despite the large differences in MWs. FTIR analysis showed that there are practically no changes in the secondary structure of the protein after either PEGylation or HESylation. *In vitro* affinity studies performed by SPR detected higher k_D values for the conjugates, mainly influenced by the shielding effect of the polymers. Analysis of the conjugate properties at high protein concentrations showed that the viscosity of HESylated anakinra was approximately 40% lower than its PEGylated counterpart at the same molar concentration. Thermal analysis experiments pointed out that the apparent melting point of anakinra increased by 2–4 K after coupling to HES or PEG, and that there is no significant difference between T_{onset} for both conjugates. Finally, the storage at 40°C over 8 weeks showed a significantly superior stability of the HESylated protein in comparison with the unmodified and the PEGylated counterparts. In conclusion, the results of this study reveal that HESylation can provide formulation advantages compared with PEGylation, especially for highly concentrated protein solutions.

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3.6. References

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Chapter IV – Freeze- dried formulations

4. Freeze-drying of HESylated IFN α -2b: Effect of HESylation on storage stability and benchmarking to PEGylation

Parts of this chapter are prepared for submission to the

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All conjugation and purification steps involved in preparing the PEGylated and HESylated proteins were performed by Sarah Bergmann, Fresenius Kabi. Georg Achleitner and his team at Fresenius Kabi Austria/Graz conducted the freeze-drying microscopy measurements. Thomas Hey and his team at Fresenius Kabi Germany/ Friedberg performed the quality control testing and the LC-MS analysis of the protein derivatives.

Abstract

The main objective of the current study was to investigate the behavior of a lyophilized, HESylated model protein in comparison with both its unmodified and PEGylated counter-parts. Attachment of polyethylene glycol (PEG) as well as hydroxyethyl starch (HES) to the N-terminus was achieved in a site-specific manner by reductive amination under mildly acidic conditions that retain the protein's secondary structure. Freeze-dried samples of the native, PEG-modified and HESylated interferon α -2b were stored for up to three months at 4 and 40°C and subsequently analyzed by a battery of analytical methods to obtain insights into conformational and colloidal changes during storage. In addition to other limitations, PEG tends to crystallize during lyophilization – a phenomenon which negatively impacts protein stability and must be suppressed by high amounts of amorphous lyoprotectants. HESylation[®] is a promising alternative to that approach for the following reasons: HES remains totally amorphous during lyophilization, facilitates a high glass transition temperature of the freeze-dried cakes, provides effective shielding to prevent protein-protein-interaction (PPI) and maintains storage stability even in the absence of additives, moving the field one step closer to excipient-free lyophilized protein formulations. Additionally, we offer some general recommendations for lyophilization of PEGylated and HESylated proteins and describe the modification of G-CSF as a second model protein to provide improved understanding of the influence of both polymers on behavior during lyophilization.

Keywords

PEGylation, HESylation, freeze-drying, phase separation, interferon α -2b, crystallization, FTIR

4.1. Introduction

Lyophilization is often implemented to increase the shelf life of labile protein and peptide solutions. Currently over 25% of approved protein products are lyophilisates. Biologics like cytokines, enzymes and peptides are known to experience limited physicochemical stability in solution, as well as short *in vivo* half-lives due to molecular weights which fall below the renal filtration threshold ($MW < 60$ kDa). Protein scaffolds, an emerging class of small, individually engineered specific binders to common targets like TNF- α , CD20 or VEGF that are based on a robust single chain polypeptide framework with remarkable conformational tolerance, represent another expanding area of research [1-3]. The efficiency of these drugs is limited by their short circulation time, which must in general be overcome by frequent injections [4]. Current state-of-the-art strategies increase plasma half-life of proteins by covalent attachment of polyethylene glycol. PEGylation leads to an increased hydrodynamic size of the conjugate, thereby slowing down kidney clearance and providing a protective coating that diminishes both the immune response and the susceptibility to degradation by proteolytic enzymes [5]. At least 10 PEGylated protein therapeutics are approved today; this technology is considered to be the gold standard for half-life extension (HLE) [6]. However, this well-established technique has some limitations, especially within the field of lyophilization [7]. In the case of Somavert[®] (PEG-hGH, Pfizer) and PegIntron[®] (PEG-IFN α -2b, Schering-Plough) an additional freeze-drying step is necessary to achieve reasonable long-term stability. Another potential drawback is that when used as a bulking agent or chemically attached to a protein, PEG tends to promote amorphous phase separation during freeze-drying, which is considered a precursor to crystallization [8, 9]. A consequence of this behavior would be a stronger tendency towards protein degradation if crystallization is not suppressed by amorphous lyoprotectants and bulking agents [9, 10]. During and immediately after the lyophilization process, this crystallization has no serious impact, but its influence increases upon storage, especially at higher temperatures [11]. Today, the most common route to overcoming crystallization is the addition of disaccharides like sucrose, which are frequently used to stabilize proteins during freeze-drying and subsequent storage in the dried state by forming hydrogen bonds that inhibit unfolding [12]. These sugars tend to remain in an amorphous state when dehydrated and can deter crystallization [13]. For freeze-dried formulations of PEGylated proteins, high sucrose-to-PEG weight ratios are required to suppress PEG-induced crystallization [8, 9, 14]. In addition, other serious limitations of PEGylation technology include PEG's non-biodegradability, the increased viscosity of PEG-conjugate solutions at higher concentrations

and the rising number of reports describing the generation of anti-PEG-antibodies; these factors have spurred both academia and industry to investigate alternative HLE approaches [7, 15, 16]. Here, we present an alternative half-life extension technology based on the coupling of hydroxyethyl starch (HES) to proteins and explore its behavior in freeze-dried formulations. HES is a biocompatible and biodegradable semi-synthetic polymer derived from a natural source (e.g. maize starch) whose use during freeze-drying is already well-known in the scientific community. However, its influence on protein stability following covalent attachment to proteins has not yet been described. HES has been previously used successfully as a cryoprotectant for red blood cells and human tissue [17-19]. When used in lyophilization, HES exhibits a higher Tg' value in solution compared to disaccharides like trehalose or sucrose, imparts excellent glass-forming properties and enables high glass transition temperatures of the solid cakes [20]. HES is generally more acceptable as a component of parenteral formulations when compared to other Tg-modifying high molecular weight carbohydrates like dextran [20] due to its lower antigenicity [21]. The use of HES as a bulking agent and/or lyoprotectant unfortunately failed for most of the reported trials involving freeze-dried biologics due to its high molecular weight and the inability to generate sufficient hydrogen bonding with the protein [22]. Garzon-Rodriguez *et al.* reported that when used in combination with disaccharides, HES (MW = 200 kDa) enabled greater stability of freeze-dried IL-11 compared to formulations which included only disaccharides or only HES. The formulations which included HES showed higher collapse temperatures and higher glass transition points, leading to improved storage stability and a potentially more economic drying process [23]. Covalent HESylation of several model proteins, on the other hand, has already proven its ability to prolong circulation [24, 25]. Additionally, site-specific HESylation of anakinra led to an improvement of both thermal and colloidal stability in comparison to the unmodified [24] and PEGylated [26] molecules. In this report, we describe how HESylation influences the stability of freeze-dried interferon α -2b as a model protein, already approved in its PEGylated version in a lyophilized formulation (PegIntron[®], Schering-Plough). We synthesized PEGylated and HESylated versions of IFN α -2b by site-specific conjugation of the polymer to the protein's N-terminus and investigated their stability upon freeze-drying and long-term storage at elevated temperatures. Additional experiments confirming the findings for IFN α -2b were conducted with HES-modified G-CSF and its PEGylated counterpart, marketed under the brand name Neulasta[®].

4.2. Materials and Methods

4.2.1. Materials

Recombinant human interferon α -2b and met-G-CSF were obtained in API quality from Sandoz (Kundl, Austria). Neulasta[®] was from Amgen (Thousand Oaks/CA, USA). Activated hydroxyethyl starch carrying an aldehyde linker at its reducing end group [24] was from Fresenius Kabi Deutschland GmbH (Bad Homburg, Germany). A 2x20 kDa branched PEG-aldehyde was purchased from Jenkem Technology (Allen/TX, USA). All other chemicals were at least of analytical grade and used as obtained.

4.2.2. Synthesis and purification of HESylated interferon α -2b

In a 6 L glass reaction vessel equipped with a thermal jacket and a blade stirrer, 30 mL of 2 M acetic acid were added to 2.4 L protein solution (protein content 5 g; 2.1 mg/mL in 50 mM sodium acetate buffer, 150 mM NaCl, pH = 4.5) to adjust the pH to 4. The solution was chilled to 4°C. 200 g of hydroxyethyl starch (Mn ~ 60 kDa) carrying a reactive propionaldehyde group were dissolved in 400 mL of 0.1 M sodium acetate buffer (pH = 4.0) pre-chilled to 5°C. 500 mL of the resulting HES reagent solution were added to the protein solution and mixed for 15 min at a stirrer speed of 100 rpm. The reductive amination reaction was started by addition of 4 g NaCNBH₃ dissolved in 100 mL water and allowed to run overnight (21 h) under moderate stirring at 5°C. In-process control samples were taken out and the reaction mixture was then frozen in aliquots at -20°C and stored at this temperature until further use. The HES-interferon conjugate was purified by cation-exchange chromatography using SP Sepharose HP material and an Äkta Purifier 100 chromatography system (both from GE Healthcare, Munich, Germany). The system was operated at room temperature with a typical flow rate of 10 mL/min. The column (XK 26/20 packed with ~130 mL resin) was first equilibrated with 5 column volumes (CV) of eluent A (20 mM sodium acetate, pH = 4.0). The reductive amination reaction mixture was diluted 5-fold with water and the pH was adjusted to pH = 4.0 using glacial acetic acid. 400 mg of conjugate were then loaded onto the column at a flow rate of 10 mL/min, followed by a washing step with 2 CV eluent A to remove the unbound, excess HES polymer. The conjugate and unreacted protein were eluted using a linear gradient from 0-100% eluent B (eluent B: 20 mM sodium acetate, 500 mM NaCl, pH = 4.0) over 20 CV. Peak fractions containing the conjugate were collected; subsequently, a buffer exchange was performed to 20 mM sodium acetate, pH = 4.5. The diafiltration step and the

final up-concentration to ~5 mg/mL (protein content) were performed by TFF using a 50 cm² PES (polyethersulfone) membrane capsule with a MWCO of 10 kDa operated on a MinimateTM benchtop system (both from Pall, Dreieich, Germany). The retentate of the TFF step was subjected to a 0.22 µm filtration step; aliquots of the filtered solution were flash-frozen in liquid nitrogen and stored at -80°C until further use. Samples for lyo-microscopy experiments were further concentrated to 20 mg/mL protein content using an Amicon Ultra 4 device (MWCO 10 kDa).

4.2.3. Synthesis and purification of PEGylated interferon α -2b

320 mL of interferon solution (2.54 mg/mL in 50 mM sodium acetate buffer, 150 mM NaCl, pH = 4.5) were subjected to diafiltration/ultrafiltration using a 50 cm² PES (polyethersulfone) membrane capsule with a MWCO of 3 kDa operated on a MinimateTM benchtop system (both from Pall, Dreieich, Germany). The protein solution was concentrated to ~10 g/L and the pH adjusted to 4.0 using acetic acid. 75 mL of the concentrated interferon solution were transferred into a 100 mL glass reactor (Mettler Toledo) and cooled down to 5°C in a Mettler Toledo Easy Max 102 system. 4 g of a 40 kDa branched PEG aldehyde (Jenkem, PALD-40 k) were dissolved in reaction buffer (0.1 M sodium acetate buffer, pH = 4.0) in a 50 mL Falcon tube to yield a 20% (w/v) solution that was slowly added to the protein solution under gentle mixing with a blade stirrer (100 rpm). The Falcon tube was rinsed with 14 mL reaction buffer and the rinsing solution also transferred into the reactor. The reductive amination reaction was started by addition of 4.5 mL of a 0.5 M NaCNBH₃ solution freshly prepared in reaction buffer. The reaction was allowed to run overnight (20 h) under moderate stirring (30 rpm) at 5°C. The reaction was quenched by addition of 20 mM glycine (final concentration) and subjected to purification after removal of in-process control samples. The PEG-interferon conjugate was purified by cation-exchange chromatography using SP Sepharose HP material and an Äkta Purifier 100 chromatography system (both from GE Healthcare, Munich, Germany). The system was operated at room temperature with a typical flow rate of 10 mL/min. The column (XK 26/20 packed with ~130 mL resin) was first equilibrated with 5 CV of eluent A (20 mM sodium acetate, pH = 4.0). The reductive amination reaction mixture was diluted 5-fold with water and 400 mg of conjugate were then loaded onto the column at a flow rate of 10 mL/min, followed by a washing step with 2 CV eluent A to remove the unbound, excess polymer. The conjugate and unreacted protein were eluted using a linear gradient from 0-40% eluent B (eluent B: 20 mM sodium acetate, 1 M NaCl, pH = 4.0) over 20 CV. Peak fractions containing the conjugate were collected and subsequently a buffer exchange was per-

formed to 20 mM sodium acetate, pH = 4.5. The diafiltration step and the final up-concentration to ~5 mg/mL (protein concentration) were performed by TFF using a 50 cm² PES (polyethersulfone) membrane capsule with a MWCO of 10 kDa operated on a MinimateTM benchtop system (both from Pall, Dreieich, Germany). The retentate of the TFF step was subjected to a 0.22 µm filtration step and the PEG-interferon α-2b conjugate solution stored at 2-8°C until further use. Samples for lyo-microscopy experiments were further concentrated to 20 mg/mL protein content using an Amicon Ultra 4 device (MWCO 10 kDa).

4.2.4. Synthesis and purification of HESylated rh-met-G-CSF

25 mg G-CSF in 9 mL of an ice-cold 10 mM sodium acetate buffer, pH = 4.0, were combined with 2 mL of a 40% (w/v) solution of hydroxyethyl starch (Mn ~ 60 kDa) carrying a reactive propionaldehyde group and dissolved in G-CSF reaction buffer (0.1 M sodium acetate pH = 5.0) in a 50 mL Falcon tube. 0.43 mL of a 0.5 M NaCNBH₃ solution, freshly prepared in reaction buffer, was added and the reaction mixture incubated at 2-8°C on a roller-mixer (20 rpm) for 20 h. The reaction mixture was subjected to purification after removal of in-process control samples. The HES-G-CSF conjugate was purified by cation-exchange chromatography using SP Sepharose HP material prepacked in 5 mL HiTrap columns and an Äkta Purifier 100 chromatography system (both GE Healthcare, Munich, Germany). The system was operated at room temperature with a typical flow rate of 5 mL/min. The HiTrap 5 mL SP HP column was first equilibrated with 5 CV of eluent A (20 mM sodium acetate, pH = 4.0). The reductive amination reaction mixture was diluted 5-fold with water and loaded onto the column at a flow rate of 5 mL/min, followed by a washing step with 2 CV eluent A to remove the un-bound, excess HES polymer. The conjugate and unreacted protein were eluted using a linear gradient from 0-100% eluent B (eluent B: 20 mM sodium acetate, 100 mM NaCl, pH = 4.0) over 20 CV. Peak fractions containing the conjugate were collected and subsequently a buffer exchange was performed to 10 mM sodium acetate, pH = 4.0 using an Amicon Ultra 15 device (10 kDa MWCO). The retentate of the ultrafiltration step (~10 mg/mL protein concentration) was subjected to a 0.22 µm filtration step, and aliquots of the filtered solution were flash-frozen in liquid nitrogen and stored at -80°C until further use.

4.2.5. Pretreatment of PEGylated rh-met-G-CSF

Commercially available PEG20-G-CSF conjugate (Neulasta[®]) is formulated in acetate buffer containing sorbitol as a tonicity modifier. Sorbitol was removed by ultrafiltration against a 10

mM sodium acetate, pH = 4.0 formulation buffer using an Amicon Ultra 4 device (MWCO 10 kDa) for benchmarking against the lyo excipient-free formulation of the HESylated protein.

4.2.6. Quality control of protein conjugates

4.2.6.1. UV-measurement

Determination of protein and conjugate concentration was performed by UV spectroscopy using the respective protein-specific extinction coefficient (IFN α -2b 279 nm: 17440 M⁻¹ cm⁻¹; G-CSF 280 nm: 15720 M⁻¹ cm⁻¹) corrected for both the absorbance of formulation buffer and potential stray light contribution at 320 nm. HES is almost transparent at this wavelength and its contribution was thus neglected.

4.2.6.2. SDS-PAGE

The purity and mass distribution pattern of the conjugate samples was tested by SDS-PAGE on 4-12% BisTris gels run in MOPS buffer (NuPAGE system, Life Technologies). Samples were processed and gels stained with Coomassie according to the manufacturer's instructions.

4.2.6.3. Size-exclusion and reversed phase chromatography

The content of soluble aggregates and unmodified protein was determined by SE-HPLC using a 7.8 x 300 mm Biosep SEC-s3000 column (Phenomenex, Aschaffenburg, Germany) equipped with the respective guard cartridge. 1x PBS with 10% (v/v) EtOH was used as eluent at a flow rate of 1 mL/min. The absence of unreacted polymer and protein was also monitored by RP-HPLC on a Jupiter C18 widepore column (5 μ m, 300A; Phenomenex, Aschaffenburg, Germany). Different methods were used for the separation of PEGylated and HESylated samples to address the differences in the hydrophilicity of the polymers. Separation of the PEG conjugate and unmodified IFN α -2b was achieved in an acidic (0.1% (v/v) TFA) water/acetonitrile system on a 4.6 x 50 mm column at a flow rate of 2 mL/min with a segmented gradient program (2-40% B in 2.5 min, 40-46% B in 2.5 min, 46% B for 2.5 min). For HES conjugations a gradient from 2-40% B in 2.5 min was used to elute the unreacted HES, followed by 40-55% B in 2.5 min to obtain separation of HES-IFN α and free protein.

4.2.6.4. AF4-MALLS measurement for the determination of the molar mass, size of the conjugates and conjugate stoichiometry

The conjugate stoichiometry was investigated by aFFFF using a Wyatt (Wyatt Technology, Dernbach, Germany) Eclipse system with triple detection (UV, RI, MALLS). A 300 mm separation chamber equipped with a 5 kDa MWCO regenerated cellulose membrane was used with a 150 mM sodium acetate, pH 4.0 running buffer at a detector flow of 1 mL/min. The components were controlled by AF4 Eclipse software (v. 2.5.1) and Astra V (v. 5.3.4.14) was used for data evaluation (both from Wyatt). The refractive index increments used were 0.185 for the protein and 0.147 for the HES compound.

4.2.6.5. Dynamic light scattering (DLS)

Dynamic light scattering was used to determine the hydrodynamic radius of the conjugate samples. Measurements were conducted in disposable cuvettes (UVette, Eppendorf, Germany) with a sample volume of 100 μ L diluted to 1 mg/mL in formulation buffer using a Zetasizer Nano Series instrument (Malvern Instruments, Herrenberg, Germany), controlled by software version 6.20.

4.2.6.6. Peptide mapping

The site-specific attachment of HES to the N-terminus of IFN α was proven by tryptic peptide mapping analyzed by RP-HPLC-ESI-MS. The conjugate sample and unmodified protein were cleaved with 7.5% (w/w) of trypsin (sequencing grade, Roche) for 18 h in phosphate buffer, pH = 8 at 37°C. Samples were reduced and denatured with 50 mM DTT and 4.5 M guanidinium chloride (final concentrations) at 100°C for 1 min prior to loading on the RP-HPLC column. The chromatographic separation was conducted on a 2 x 150 mm Jupiter C18 column (5 μ m, 300 Å) in a segmented gradient (0-7 min, 3% B; 7-15 min, 3-10% B; 15-45 min, 10-50% B; 45-65 min, 50-70% B; 65-70 min, 70% B; 70-90 min, 3% B) with eluent A (0.1% (v/v) formic acid, 0.01% (v/v) TFA in water) and eluent B (0.1% (v/v) formic acid in acetonitrile). Elution of the peptides was monitored at 214 nm. Peak identification and peptide assignment was conducted based on the MS signal obtained from a MicrO-TOF system coupled to an ESI device (Bruker Daltonik, Bremen, Germany).

4.2.7. Freeze-Drying Microscopy

For freeze-drying microscopy the lyostat 2 microscope (BTL, Winchester, UK) controlled by Linksys 32 software (Linkam, Surrey, UK) was used. For the preparation of samples, a 15 mm round quartz slide was placed in the sample holder on the silver block of the freezing stage. This block was coated with a drop of silicon oil to improve heat transfer from the silver block to the glass cover slide. Approximately 2 μL of test solution were pipetted onto the slide and subsequently covered by a second 9-mm cover glass slide. Precision-cut spacers (height 0.025 mm) were located in-between both slides to maintain a constant sample height. The cooling rate used throughout this study was 5°C/min. The heating rate was 1.0°C/min in the temperature range of interest to allow a representative investigation of the first structural changes in the product. The pressure in the freeze-drying chamber was below 1 Pa (0.01 mbar) when the sample was dried. Pictures of the respective sample were taken in 15-sec intervals by using a digital camera system mounted on top of the microscope (200-fold magnification). Pictures were then analyzed for onset of collapse, and full collapse temperature, using the Linksys 32 software. The onset of collapse was defined as the temperature at which first gaps and fissures were visible in the frozen sample adjacent to the sublimation interface. Full collapse was defined as the temperature at which a full loss of structure (i.e. big holes, melting) could be determined.

4.2.8. Sample preparation for lyophilization

Native, PEGylated and HESylated IFN α -2b were rebuffered into 25 mM citrate buffer at pH 5.0 using 70 mL Slide-A-Lyzer[®] dialysis cassettes (Thermo Scientific, Schwerte, Germany). The protein concentration was determined by UV-VIS spectroscopy at 280 nm using an extinction coefficient of $\epsilon = 0.9051$ (provided by the original protein supplier).

4.2.9. Tg' measurement

The Tg' was determined by differential scanning calorimetry on a Netzsch DSC 204 Phönix[®] (Netzsch, Selb, Germany). 20 μL of each formulation was hermetically sealed in 40 μL aluminum pans, cooled from 20 to -70°C at a rate of 10°C/min and subsequently heated up to 20°C at a rate of 10°C/min to detect the glass transition temperature of the frozen solution.

4.2.10. Freeze-drying protocol

The freeze-drying process was performed in a Martin Christ Epsilon 2-6D pilot-scale freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) using the following freeze-drying protocol. 2R vials (Vetter, Ravensburg, Germany) were filled with 1 mL sample volume. After shelf loading the vials were frozen by cooling at a rate of 1°C/min from 20 to -45°C and holding at the final temperature for 90 min. Primary drying was performed at 0.1 mbar by implementing a heating ramp from -45 to -25°C at a rate of 0.1°C/min and holding the final temperature for 30 h. Secondary drying was performed by implementing an equivalent ramp up to 20°C and holding the system at 0.01 mbar for 15 h. The vials were flooded with gaseous nitrogen, stoppered with Teflon-coated stoppers (West Pharmaceutical Services, Eschweiler, Germany) at 800 mbar and afterwards manually sealed with 13 mm flip off seals (West Pharmaceutical Services, Eschweiler, Germany).

4.2.11. T_g and degree of crystallization

The glass transition temperature of the freeze-dried cakes was determined by DSC measurement. Under a pressurized, air-flooded glove box with a relative humidity between 5-10%, a sample amount of 1-20 mg was weighed in aluminum pans and hermetically sealed. For DSC measurement the pans were heated from 10 to 110°C, cooled to the starting point and heated again to 180°C with a constant heating rate of 10°C/min. Due to structural relaxation of the cakes (T_g overshoot), the samples had to be heated twice to determine the correct glass transition temperature. Additionally, the DSC thermograms imply further information about the crystallization processes. Therefore, the enthalpy of crystallization as represented in J/g, was determined by the AUC of the crystallization peak.

4.2.12. Residual moisture

The moisture content of the cakes was measured by a coulometric Karl-Fischer-Titrator (KF 373, Metrohm GmbH & Co, Filderstadt, Germany).

4.2.13. Turbidity measurement

The turbidity in 2 mL sample volumes was determined at 860 nm and given in formazine nephelometric units (FNU) by using a NEPHLA turbidimeter (Dr. Lange, Düsseldorf, Germany).

4.2.14. Particle count - Light obscuration

According to Ph. Eur. and USP specifications, particle analysis was performed by light obscuration using a PAMAS SVSS-C particle counter (PAMAS, Rutersheim, Germany) [27]. Particles with a size of 1, 10 and 25 µm were quantified after a rinse volume of 0.5 mL in three runs of 0.3 mL with an emptying rate and rinse rate of 10 mL/min.

4.2.15. Monomer recovery – Size exclusion chromatography

HP-SEC was performed on a Spectra System P2000 (Thermo Scientific, Germany) equipped with a TSK guard column and a TSKgel G3000 SWxl (Tosoh Bioscience GmbH, Stuttgart, Germany). After an injection of 50 µg protein, each run was performed by an isocratic elution of the mobile phase, containing 25 mM citrate, 150 mM NaCl, 1% diethylene glycol, 10% ethanol, pH 5.0 at 0.5 mL/min over 45 min followed by UV detection at 280 nm (buffer composition adapted from Kusterle *et al.* [28]).

4.2.16. Conformational changes - FTIR

FTIR spectroscopy was investigated on the Bruker Tensor 27 FTIR with the Aquaspec cell (Bruker Optics, Ettlingen, Germany). The sensor was cooled with liquid nitrogen and flushed with a constant gaseous nitrogen flow. The samples were analyzed at a protein concentration of 3 mg/mL in 40 scans against pure formulation buffer, which was used for background subtraction. The curves were normalized by vector normalization using the OPUS Software to detect changes in secondary structure in the second diversion of each spectrum.

4.3. Results

4.3.1. Preparation and quality control of the polymer modified interferon samples

The SDS-PAGE analysis conducted on conjugate samples before and after chromatographic purification reveals information on the conjugation turnover, the conjugate purity and potential changes in the mass distribution pattern due to purification (**Fig. 1**). The comparison of the crude reaction mixtures on the left with the purified samples on the right proves that unreacted protein is efficiently depleted by the cation exchange chromatography step, since the

low molecular weight band at ~15-20 kDa is no longer detectable in the purified conjugates. Furthermore, it can be seen that di-PEGylated protein was also removed during the purification. The mass distribution pattern of the polydisperse HES conjugate, seen as a broad smear on the gel, remains unchanged after purification.

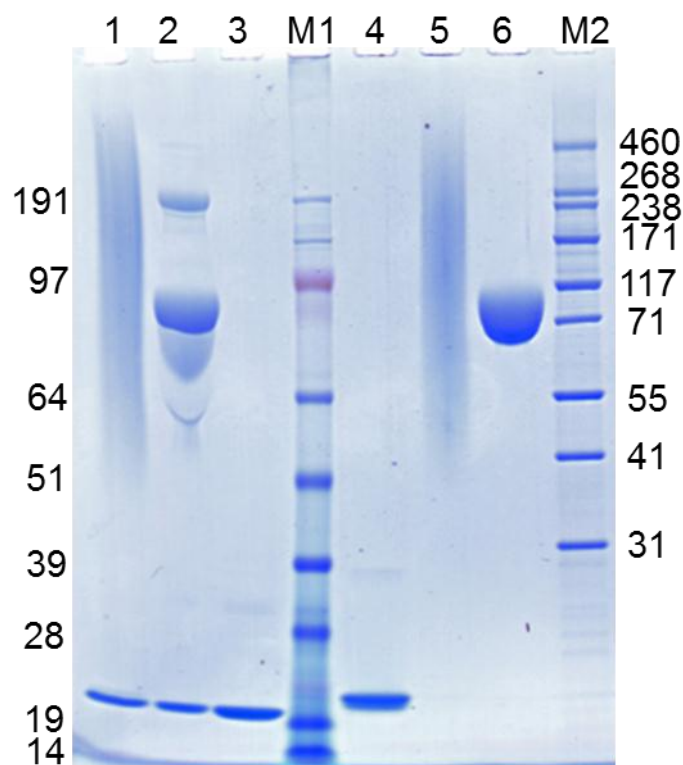


Figure 1: SDS-PAGE analyses, 1: HES-IFN α reaction mix (non-red.) 2: PEG-IFN α reaction mix (non-red.) 3: IFN α (non-red.) M1: SeeBlue[®] Plus2 Prestained Standard 4: IFN α (reducing) 5: HES-IFN α purified (reducing) 6: PEG-IFN α purified (reducing) M2: HiMark[®] Standard

These observations are also confirmed by the chromatographic analyses shown in Fig. 2. For the PEGylated sample an additional peak, eluting just before the main product, is visible in the reaction mixtures; this peak is removed during purification and no longer appears in the final product as assessed by both RP-HPLC and SE-HPLC. In the RP-HPLC analysis it can also be seen that the unreacted polymer is removed in the ion exchange step, since a tiny peak eluting in the first part of the gradient is no longer visible in the purified samples. Only negligible amounts of free protein, which cannot be quantified using the methods applied here, are present in the final product. The SEC analysis reveals the presence of only minor amounts of soluble aggregates (< 0.5%). The data analysis is slightly impaired by peak fronting of the HES conjugate, caused by its broader mass distribution and the presence of larger HES molecules conjugated to the protein. Some of these molecules fall outside of the separation range

of the SEC column used here and led to a “pseudo” peak at the void volume that could also be assigned as a soluble aggregate. But re-analysis of the samples on a column with a broader separation range (Superose 6, GE Healthcare) unambiguously confirmed the absence of aggregates in all conjugate preparations (data not shown).

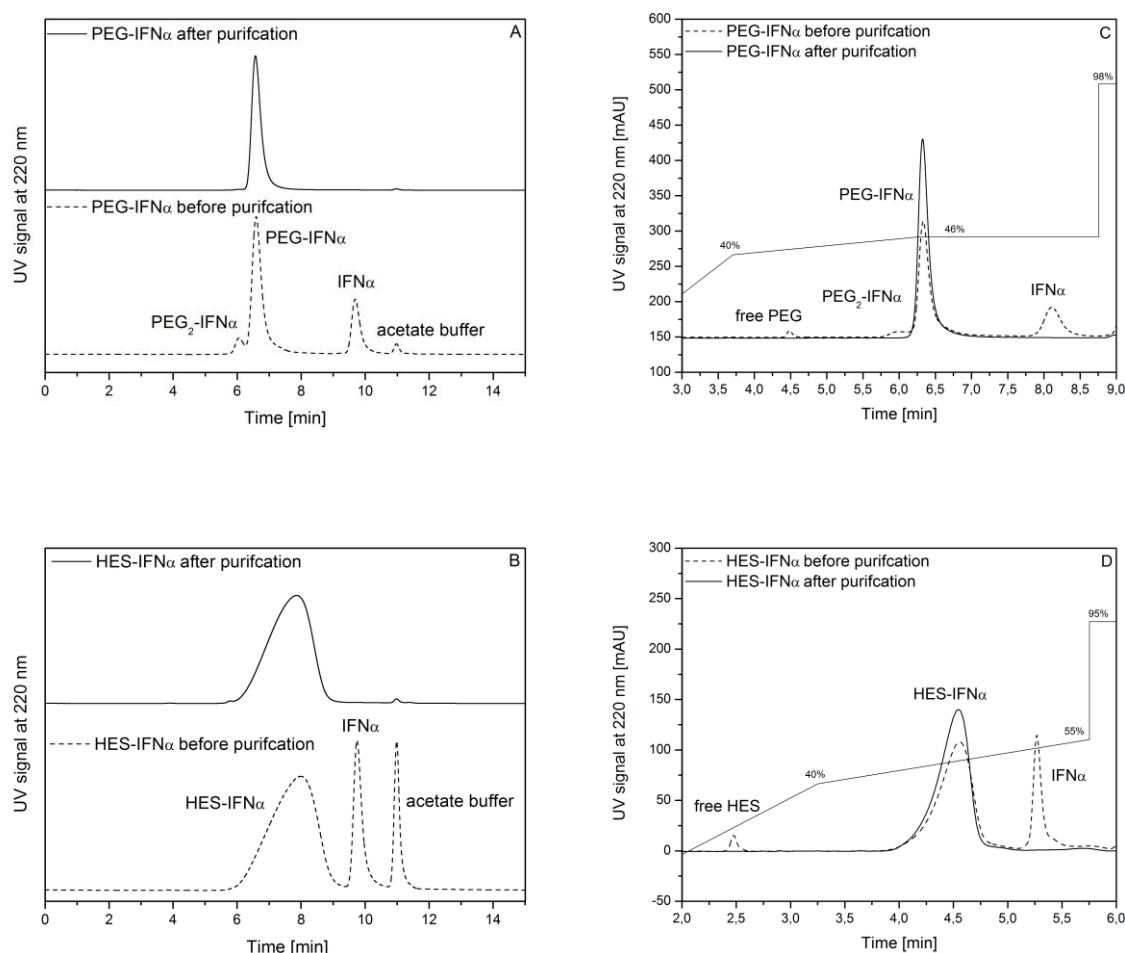


Figure 2: A: SEC analyses of PEG-IFN α , B: SEC analyses of HES-IFN α , C: RP-HPLC analyses of PEG-IFN α and D: RP-HPLC analyses of HES-IFN α before and after purification

A A 2x20 kDa branched PEG was chosen for the modification of IFN α -2b since it is used in a number of approved drugs, including PEGasys[®], a randomly mono-PEGylated IFN α -2a. In order to obtain an HES conjugate having a comparable biologic effect it was necessary to use an HES polymer of higher molecular weight to address the conformational and flexibility differences between the two polymers: While the linear PEG chains adopt an extended coil conformation due to their high flexibility, the HES molecule has a more condensed, globular,

tree-like shape since it contains rigid glycosidic bonds and a large number of branches. The hydrodynamic radius of the resulting conjugates was comparable as shown by DLS measurements. As is to be expected, the polydispersity was slightly higher for the HESylated sample (see **Table 1**).

Table 1: Weight average molar mass (Mw) and polydispersity index (PDI) for the activated polymers, native protein and conjugates.

Sample	Hydrodynamic radius [nm] ^b	PDI ^b	Conjugate		Polymer		Protein		Polymer/ Protein Ratio
			Mw by	Mn by	Mw by	Mn by	Mw by	Mn by	Modifier in conjugate/ modifier before conjugation ^a
			RI/MALLS [kDa] ^a	RI/MALLS [kDa] ^a	RI/MALLS [kDa] ^a	RI/MALLS [kDa] ^a	RI/MALLS [kDa] ^a	RI/MALLS [kDa] ^a	
IFN α -2b	2.1	0.26	n.a.	n.a.	n.a.	n.a.	19.8	19.2	n.a.
PEG-IFN α	6.1	0.21	68.4	65.8	47.9	46.1	20.5	19.7	1.14
HES-IFN α	5.9	0.54	101.2	85.8	82.0	61.4	19.2	18.9	1.01
Activated PEG	4.7	0.23	n.a.	n.a.	42.8	40.4	n.a.	n.a.	n.a.
Activated HES	4.7	0.55	n.a.	n.a.	81.0	60.5	n.a.	n.a.	n.a.

^a determined by AF4-MALLS^b determined by DLS

Table 1 also shows the results of aFFFF analysis followed by triple detection. The combination of light scattering data, refractive index measurements and UV absorption at 280 nm allows a virtual split of the conjugate into its single components, thus yielding information on its stoichiometry. It should be noted that the molecular weight determined for the protein is close to the theoretical value. For HESylated IFN α -2b the molecular weight calculated for the polymer fraction is almost identical to the value determined for the HES reagent as assessed before conjugation, clearly suggesting a 1:1 stoichiometry. The value is slightly higher than 1 in the case of the PEG conjugate, although the other analysis methods employed clearly show a mono-PEGylated protein and successful depletion of di-PEGylated species. It is also evident that the Mw deduced for the protein fraction is slightly higher than for the other conjugates. A potential explanation for this phenomenon may be the presence of some loose conjugate dimers leading to a virtual increase in the average molecular weight. The conjugation site of the polymer on the protein was determined by peptide mapping for the HESylated sample (**Fig. 3**). The RP-HPLC/ESI-MS analysis of the tryptic digest showed the complete disappearance of the N-terminal peptide peak in the conjugate sample. The polydisperse nature and high hydrophilicity of the HES polymer resulted in a broad and front-shifted peak representing the HESylated N-terminal peptide T1, while all other peaks remain unchanged, clearly pointing towards the N-terminus as the predominant modification site.

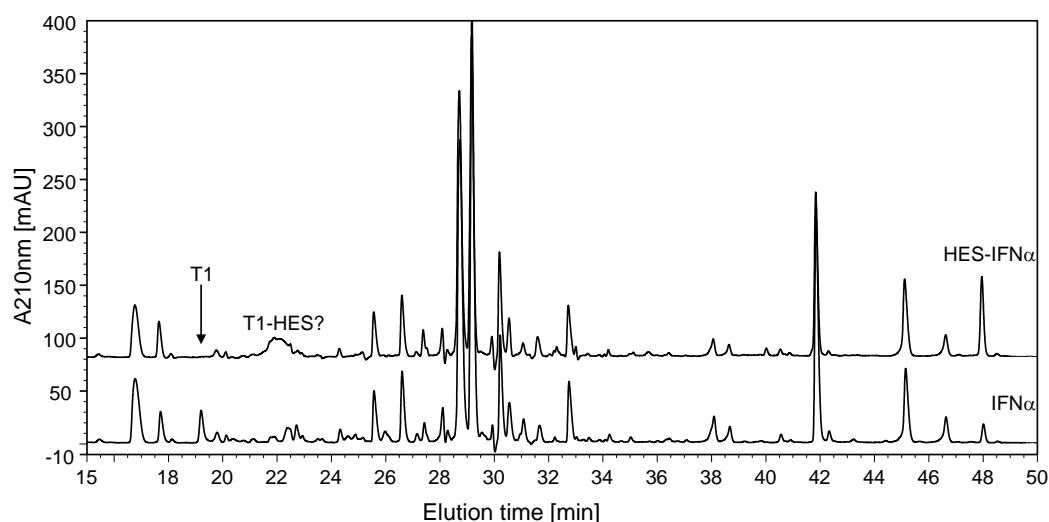


Figure 3: Peptide Mapping of HES-IFN α

4.3.2. Lyomicroscopy

PEGylated and HESylated interferon as well as the PEGylated and HESylated version of G-CSF were characterized by lyomicroscopy to investigate general aspects of collapse temperature, cake appearance and density, which are mainly influenced by the attached polymer. Commercially available PEGylated G-CSF (Neulasta®), under the selected conditions, begins to collapse at -23.2°C, resulting in a final collapse temperature of -22.3°C. Cake appearance and density showed a sub-optimal cake structure with a more liquid-like character (**Fig. 4**). In contrast, HESylated G-CSF showed a much higher onset in collapse temperature at -11.1°C resulting in a final T_c of -10.6°C. Cake appearance and density are drastically improved upon HESylation.

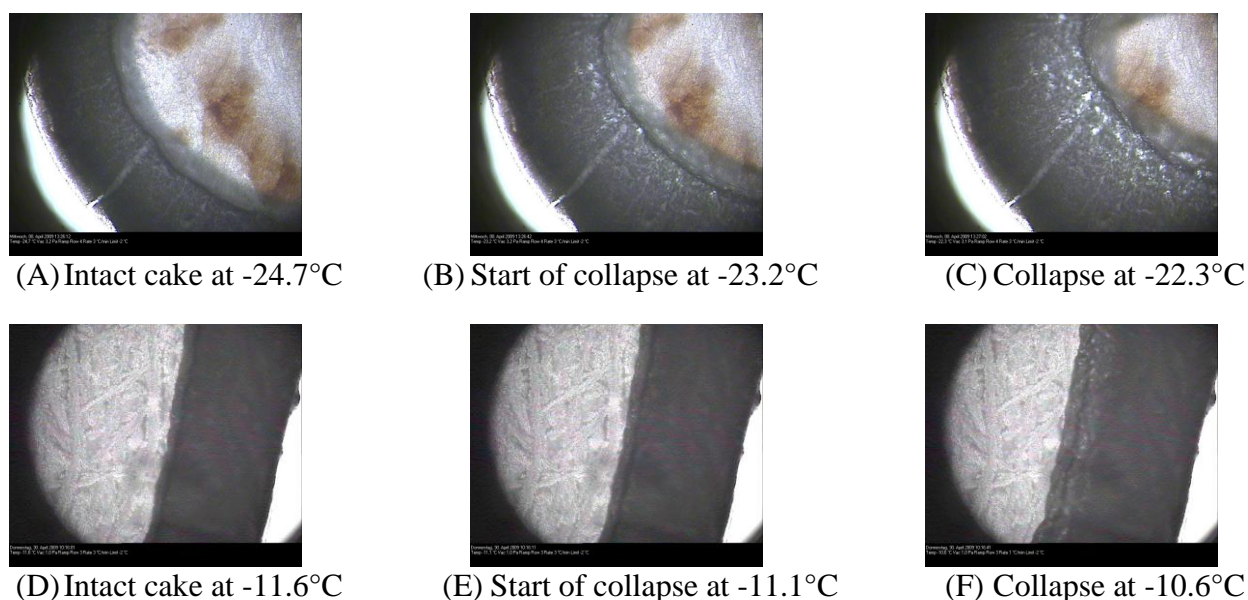


Figure 4: Freeze-drying microscopy of PEG- (A-C) and HESylated G-CSF (D-F)

Comparable trends are given for PEGylated and HESylated IFN α . The PEGylated protein started to collapse at a much earlier point ($T_{\text{conset}} = -36.1^{\circ}\text{C}$ and $T_c = -35.4^{\circ}\text{C}$) than HES-IFN α , which showed a T_{conset} of -23.4°C and a T_c of -22.7°C under the chosen conditions (**Fig. 5**).

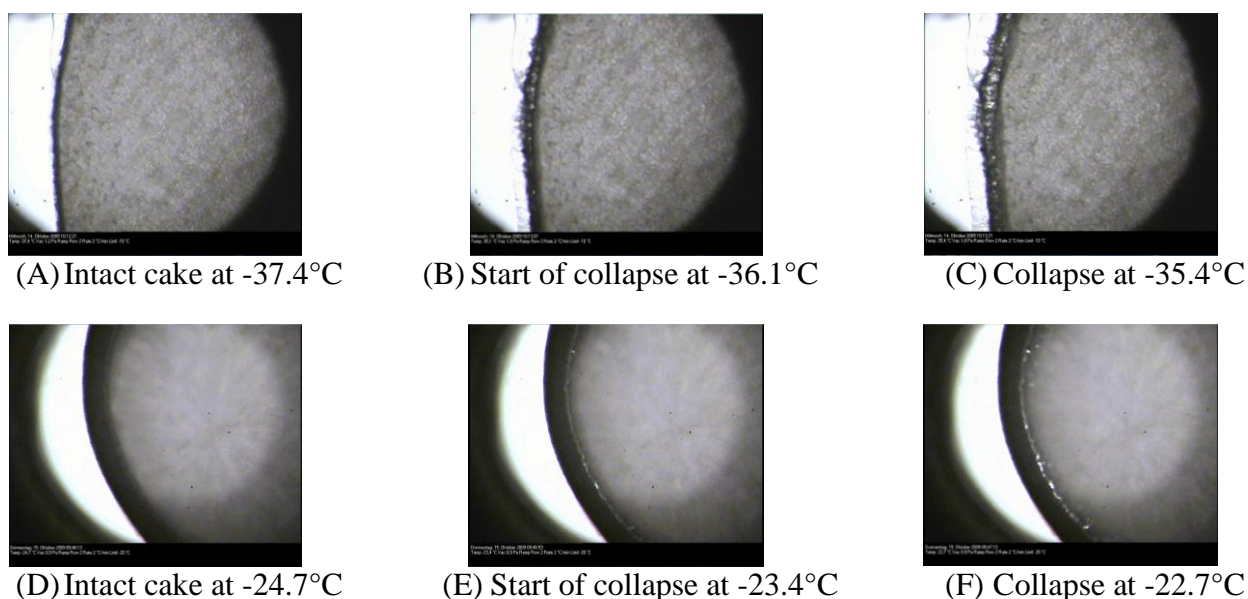


Figure 5: Freeze-drying microscopy of PEG- (A-C) and HESylated IFN α -2b (D-F)

4.3.3. Thermal analysis of the frozen formulations

The thermal properties of the frozen solutions were further characterized by differential scanning calorimetry to determine the glass transition temperatures of the maximally freeze-concentrated matrix; these temperatures represent a critical product temperature associated with collapse during freeze-drying. **Table 2** summarizes the Tg' values of the chosen formulations. Obviously, the Tg' values differ significantly from the obtained collapse temperatures, caused mainly by the difference in protein concentration (see Materials and Methods).

Sample	Protein conc. [%m/v]	Sucrose conc. [%m/v]	Tg' [°C]
Native IFN α	0.1	0	*
	0.1	2.5	-34
	0.1	5	-33
PEGylated IFN α	0.1	0	*
	0.1	2.5	-35
	0.1	5	-33
HESylated IFN α	0.1	0	*
	0.1	2.5	-31
	0.1	5	-33

Table 2: Thermal analysis of the frozen formulations before lyophilization, * not detectable

4.3.4. Optical evaluation of the freeze-dried samples

Figure 6 illustrates the cakes after freeze-drying. Vials A, D and G represent native, PEGylated and HESylated IFN α , respectively, lyophilized in the absence of sucrose. The native protein was heavily affected by cake shrinkage due to the absence of lyoprotectants.



Figure 6: Optical evaluation of the freeze-dried formulations; (A) IFN α without sucrose (B) IFN α and 2.5% sucrose (C) IFN α and 5% sucrose (D) PEG-IFN α without sucrose (E) PEG-IFN α and 2.5% sucrose (F) PEG-IFN α and 5% sucrose (G) HES-IFN α without sucrose (H) HES-IFN α and 2.5% sucrose (I) HES-IFN α and 5% sucrose

4.3.5. Cake properties after lyophilization and storage

4.3.5.1. Glass transition temperature and degree of crystallization

Immediately after freeze-drying the native and PEGylated IFN α showed for the sucrose based formulations a glass transition temperature in a range between 69 and 74°C, which remained more or less constant during storage. Due to the high T_g of the HES part in the molecule, the glass transition temperature exceeded 110°C for the sucrose free formulation of HES-IFN α and was substantially lowered by the addition of 2.5 and 5% disaccharide to 67°C. The PEG-induced crystallization was fully suppressed using 5% m/v sucrose (**Fig. 7**). During storage the degree of crystallization for PEG-IFN α without and in combination with 2.5% sucrose slightly increased over time, especially for the samples stored at 40°C.

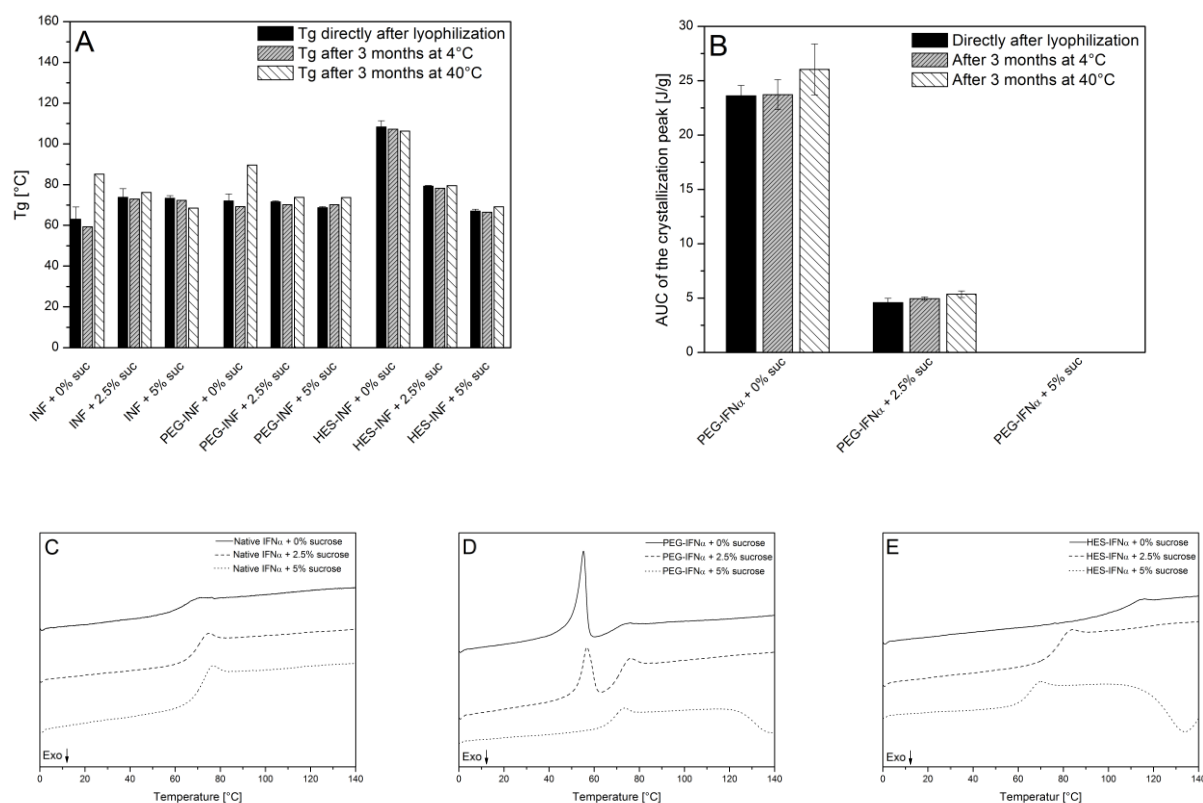


Figure 7: Tg measurement (A): black bars represent the initial Tg after freeze-drying; the grey-dashed ones after 3 months storage at 4°C and the white- dashed ones after 3 months storage at 40°C; AUC of the crystallization peak (B): black bars represent the initial value after freeze-drying; the grey-dashed ones after 3 months storage at 4°C and the white- dashed ones after 3 months storage at 40°C, Representative thermogramms taken immediately after freeze-drying (C,D,E)

4.3.5.2. Residual moisture

Table 3 summarizes the residual moisture data of the lyophilized cakes immediately after lyophilization and after 3 months storage at 4 or 40°C. The addition of sucrose substantially lowers the residual moisture. In general, both conjugates showed, without the addition of sugar, lower initial moisture contents in contrast to the unmodified protein. In a direct comparison of PEG and HES, the coupling of HES led to lower values lying in the fact, that HES has a better drying behavior due to the sugar based scaffold of the molecule. In contrast, PEG is highly hydrophilic and can coordinate a higher number of water molecules, which are apparently harder to remove by freeze-drying.

Formulation	Native IFNα	PEGylated IFNα	HESylated IFNα
0% sucrose	Residual moisture [%]	Residual moisture [%]	Residual moisture [%]
T0	3.6 \pm 0.2	2.7 \pm 0.2	2.3 \pm 0.1
T3 at 4°C	4.1 \pm 0.4	3.0 \pm 0.4	1.3 \pm 0.2
T3 at 40°C	1.9 \pm 0.4	1.1 \pm 0.0	1.1 \pm 0.1
2.5% sucrose			
T0	1.0 \pm 0.3	1.5 \pm 0.1	1.1 \pm 1.1
T3 at 4°C	1.2 \pm 0.3	1.2 \pm 0.1	0.7 \pm 0.1
T3 at 40°C	0.8 \pm 0.1	0.9 \pm 0.1	0.6 \pm 0.0
5% sucrose			
T0	1.0 \pm 0.1	1.0 \pm 0.1	1.2 \pm 0.2
T3 at 4°C	1.0 \pm 0.1	0.7 \pm 0.1	0.9 \pm 0.2
T3 at 40°C	0.8 \pm 0.0	0.6 \pm 0.0	0.9 \pm 0.1

Table 3: Residual moisture contents immediately and after storage for 3 months at 4 or 40°C; Key: T0: immediately after freeze-drying, T3: 3 months storage at 4 or 40°C

4.3.5.3. Conformational stability after lyophilization and storage

FTIR analyses of the conjugates provided two essential insights. First, it helps to illustrate how PEGylation and HESylation alter the secondary structure after polymer coupling (**Fig. 8**) and second, it shows how the formulation influences conformational stability after freeze-drying and during storage. Surprisingly, significant changes in secondary structure upon freeze-drying and storage could not be detected for native protein and both conjugates, indicating high conformational stability (data not shown).

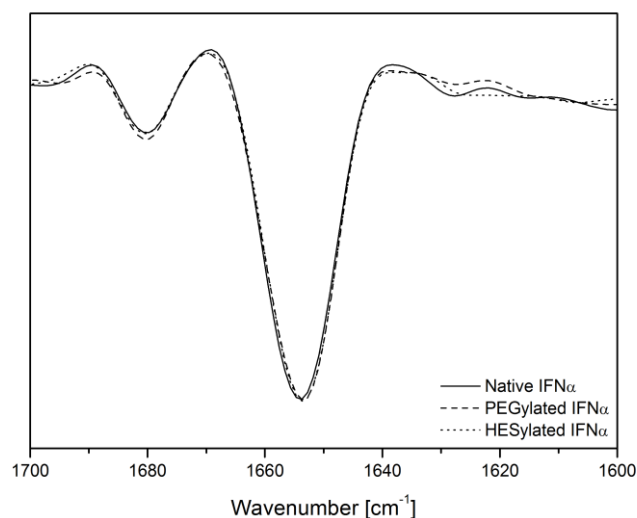
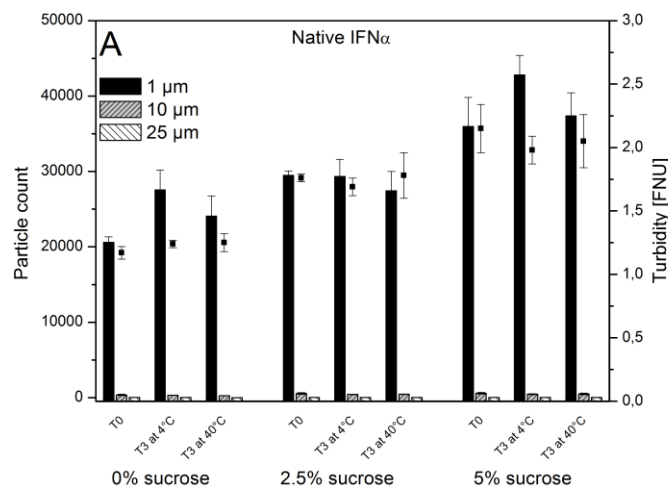


Figure 8: Area-normalized, second-derivative amide I spectra of native (solid line), PEG- (dashed line) and HESylated IFN α (dotted line) of the liquid samples

4.3.6. Colloidal stability after lyophilization and storage

4.3.6.1. Turbidity and particle counts

Turbidity values and particle counts are illustrated in **Figure 9**. The attachment of PEG or HES to the protein prevented the formation of particles due to a shielding effect independent of the type of polymer used. Samples of both conjugates showed significantly lower initial particle counts compared to those of the native IFN α . The turbidity measurement correlates well to the total particle counts.



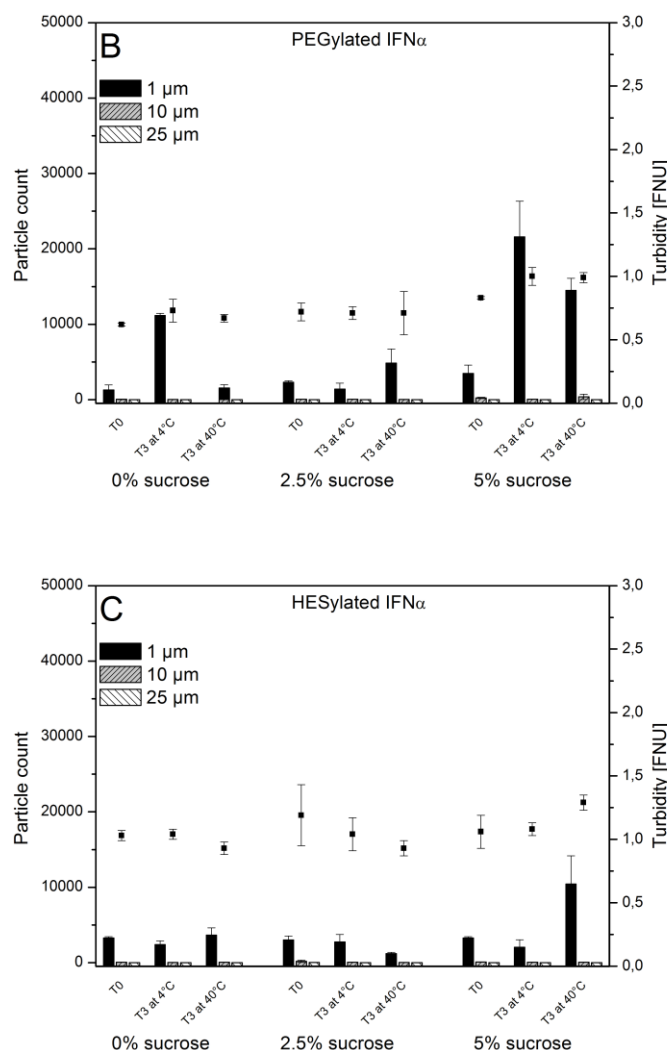


Figure 9: Turbidity and particle count; (A) native IFN α ; (B) PEGylated IFN α and (C) HESylated IFN α ; Key: T0: immediately after freeze-drying, T3: after 3 months storage at 4 or 40°C

4.3.6.2. Monomer recovery and soluble aggregates

The monomer content and the generated level of soluble aggregates during storage at 4 or 40°C as a function of the formulation's sucrose content was determined by HP-SEC (**Fig. 10**). The data show that the sucrose-to-polymer weight ratio and the chosen storage conditions have the greatest influence on the formation of aggregates. Neither native nor HESylated IFN α showed a dramatic decrease in monomer recovery, nor were soluble aggregates formed, even at 40°C. Taking the data from soluble and insoluble aggregates together, it is even possible to formulate HESylated interferon α -2b without any sugar. In contrast, the colloidal stability of PEGylated interferon α -2b is highly dependent on the sucrose-to-PEG weight ratio and storage temperature. For example, storage of lyophilized PEGylated IFN α at 4°C without

the addition of sucrose caused soluble aggregates to increase only slightly, from 1.1 to 1.2%. At 40°C, the level of soluble aggregates increased remarkably, from 1.1 to 6.9%, indicating the strong influence of storage temperature. The addition of sucrose substantially prevented aggregation. Increasing the concentration of sucrose from 2.5% to 5% led to a significant decrease in the level of soluble aggregates, from 5.5% to 1.3%, for samples stored at 40°C. Interestingly, losses in monomer recovery correlate quite well to the degree of crystallization (see supplementary data). When crystallization of PEG is not suppressed by amorphous lyoprotectants, the PEGylated protein will rapidly degrade during long-term storage.

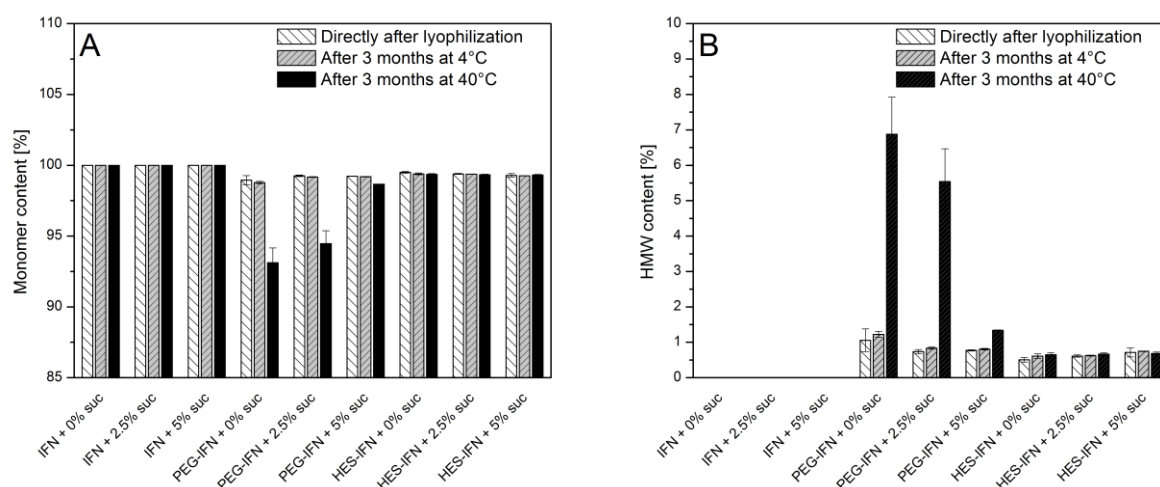


Figure 10: HP-SEC measurement to quantify monomer content (A) and the level of high-molecular weight species (HMW; B) after 3 months at 4 or 40°C

4.4. Discussion

Today, approval of conjugated biologics requires site-specific chemistry to obtain homogeneous batches of single isomers [6]. Targeting the N-terminus to obtain a well-defined product can be performed by reductive amination under mildly acidic conditions (e.g. at a pH of 4- 5). The reaction takes place preferentially at the N-terminal amino group, which typically displays a lower pKa value of ~8 compared to ~10 for the ϵ -amino groups of lysine residues [29-31]. In the first step of the conjugation a labile Schiff's base is formed, which is subsequently reduced by sodium cyanoborohydride to a stable secondary amine [32]. This approach worked exceptionally well for conjugations involving interferon α -2b; peptide mapping and aFFFF-MALLS analysis shown above provide proof of site-specific mono-conjugation. Furthermore, our results are in good agreement with previously reported variants of PEGylated IFN α -2b carrying a range of PEG derivatives at the N-terminus [28]. The resulting conjugates were of excellent purity, with only trace amounts of unmodified protein and aggregates de-

tected in the liquid formulations that served as a starting point for lyophilization experiments. Evaluation of the collapse temperature of frozen formulations is a critical tool for the selection of appropriate lyophilization parameters [33, 34]. Using lyomicroscopy, the freezing and annealing step can be simulated and visually tracked to assess performance of the samples under the selected lyo conditions [33]. The general goal is to maintain as high a collapse temperature as possible [20]. The obtained T_c is directly related to the maximally allowed product temperature, which must be several degrees below T_c to maintain an acceptable appearance of the dried cake [34]. With this context, PEGylated and HESylated versions of two pharmaceutically relevant proteins were tested for their performance during freezing and thawing in an adjusted lyo cycle. The presence of hydroxyethyl starch in the conjugate led to an extraordinarily high collapse temperature of the frozen solution; the precise T_c value depends on the amount of HES present in the formulation. The sugar-based HES scaffold also improves cake appearance and density, whereas PEG, when conjugated to the same protein and yielding comparable conjugate hydrodynamic size, does not. The very high collapse temperature of HES enables a more economical and robust lyophilization process. In general, freeze-drying is a time- and cost-intensive process; therefore, the target product temperature, which is highly dependent on the collapse temperature, should be as high as possible. For example, an almost 1°C increase in product temperature lowers the time for primary drying by about 13% [34]. As a proof of concept, formulations of PEGylated and HESylated IFN α were compared to the unmodified protein in terms of conformational and colloidal stability after freeze-drying and storage at elevated temperatures (4 and 40°C) for 3 months. With the exception of the formulation consisting of unmodified protein and no sucrose, the freeze-drying protocol led to pharmaceutically elegant cakes, indicating that primary drying occurred under the critical collapse temperature of the frozen solutions. The determined T_g' values correlated well to reported data for other sucrose-based formulations [20]. In general, HES solutions are characterized by high T_g' values, ranging from -12 to -17°C depending on the HES molecular weight, the degree of substitution and the HES-to-disaccharide ratio [13, 35, 36]. Our results, however, demonstrate only a slight increase in the T_g' value for the HESylated protein. The most likely reason for this is that the total amount of HES in a formulation of 1 mg/mL protein concentration is rather low, having almost no effect on T_g' and process settings. To ensure adequate storage stability after drying, the T_g of the dry cake should be as high as possible, preferably at least 20°C above storage temperature [13]. Our data indicate that this condition has been easily met, as we report T_g values of at least 60°C up to ~110°C – well above ambient temperature. Although it is quite common for sucrose-based cakes with

residual moisture in the range of 1% to have Tgs of about 65°C [37], some of our data are remarkable. HES is obviously capable of acting as a Tg modifier when covalently bound to the protein. With no sucrose added, HESylated interferon α -2b has a Tg of ~110°C after freeze-drying. The addition of 2.5% or 5% sucrose inhibits this particular effect of HESylation, leading to Tgs of 75°C and 65°C, respectively. In addition, native and HESylated IFN α did not show any tendency toward crystallization. Hydroxyethyl starch is based on a carbohydrate scaffold, and thus acts as a completely amorphous bulking agent during freeze-drying, in a similar fashion to dextran [23, 33]. For PEGylated IFN α , we observed a different situation entirely. PEG is prone to rapid crystallization during lyophilization, due to its tendency to promote phase separation into polymer-rich and sugar-rich phases, a precursor for crystallization [11]. Therefore, high sucrose-to-PEG weight ratios (≥ 5) are required to fully suppress PEG-induced crystallization. If crystallization occurs during storage, it is in most cases accompanied by an increased propensity for protein degradation [11]. In general, polymer conjugation under mild reaction conditions should have no effect on the secondary and tertiary structure of the protein [38]. Using CD measurement, Kinstler and colleagues observed no effect of site-specific PEGylation of G-CSF on the protein conformation; these results were supported by FTIR studies from Rajan *et al.* [38]. Our results confirm these findings; we recently reported that neither PEGylation nor HESylation altered the secondary structure of rh IL-1ra (anakinra) [26]. In this study, the 2nd derivative FTIR spectra were obtained after storage of the freeze-dried samples. Furthermore, native, PEGylated and HESylated IFN α underwent lyophilization and subsequent storage without evidence of any changes, indicating high conformational stability. Colloidal stability of IFN and its conjugates was assessed by turbidity measurement, particle counting and monomer recovery by HP-SEC [39, 40]. Typically, protein PEGylation reduces aggregation by physically separating monomers from one another and therefore leads to reduced protein-protein interaction [41, 42]. The ability of PEGylation to prevent PPI is not unique; this effect can also be obtained via HESylation [26]. In this study, PEGylated and HESylated IFN α showed significantly lower particle counts after reconstitution of the freeze-dried samples compared to that of the native protein. As mentioned above, turbidity measurement can be used as a predictive marker for changes in a sample's aggregate status [27]. However, the turbidity measurement can be influenced by excipients or high protein concentration and high values do not necessarily reflect high numbers of proteinaceous particles [43]. Polymers can also scatter light and lead to high background turbidity [44, 45]. In our studies we found that HESylation leads to higher initial turbidity values compared to those of both the native and PEGylated protein. However, this value is not a reliable

marker of higher numbers of aggregates, which was investigated by LO and HP-SEC measurements. As mentioned above, PEG-induced crystallization fosters protein degradation if it is not suppressed by some means, such as high sucrose-to-PEG weight ratios. Stability studies from Bhatnagar *et al.* regarding freeze-dried PEGylated human growth hormone found the lowest colloidal stability in samples which were lyophilized with low sucrose to PEG weight ratios [11]. We can confirm those findings for PEG-interferon. In particular, even the addition of 2.5% sucrose was insufficient to prevent aggregation of PEGylated IFN α . Therefore, a concentration of at least 5% sucrose must be included to prevent protein degradation induced by PEG-driven crystallization. In contrast, HESylation drastically improved the colloidal stability of the conjugate, leading to reduced monomer loss and aggregate formation. This can be explained by two facts: first, HES remained totally amorphous during lyophilization. Second, when covalently attached to a protein, HES provides a protective shield that prevents PPI. Additionally, for a sucrose-free lyophilisate of HESylated IFN α , a very high T_g will also most likely lead to long-term storage stability even at greatly elevated storage temperatures. Finally, a sucrose-free, freeze-dried formulation allows for a very high protein load, which is extremely attractive for further manufacturing steps, such as integration with drug delivery systems.

4.5. Conclusion

Site-specific mono-modification of the model protein interferon α -2b with PEG or HES was achieved by reductive amination. Conjugate preparations of high purity were obtained by cation exchange chromatography without altering the protein's secondary structure. Some fundamental questions concerning cake appearance and density were clarified for both polymers and illustrated using conjugated IFN α -2b and G-CSF. Upon freeze-drying of PEGylated proteins, the use of high sucrose-to-PEG weight ratios is necessary to prevent protein degradation during long-term storage. In contrast, HESylation was found to be an attractive alternative PEGylation, overcoming several significant formulation challenges associated with manufacturing of lyophilized PEGylated proteins. We demonstrate HESylation of IFN α as an example of chemical coupling of a biocompatible polymer with excellent lyophilization properties; the conjugate remained completely amorphous throughout lyophilization. The high glass transition temperature of pure HES facilitated an elevated T_g of the HESylated protein during freeze-drying, which can be an essential feature for manufacturing steps characterized by high residual moisture content, such as spray or vacuum drying. Additionally, HESylation effectively shielded the conjugate to prevent PPI and improve conjugate colloidal stability. Lyophilized

formulations with no or low concentration of sucrose, as tested in this study, could be used to up-concentrate protein solutions or permit a high protein load; both of these features would be attractive for further manufacturing steps, like bulk drying of API or loading into drug delivery systems. In conclusion, this study reveals that HESylation offers a number of advantages over PEGylation with regards to the development of freeze-dried formulations of polymer-protein conjugates. In particular, higher collapse and glass transition temperatures, as well as an amorphous nature which maintains storage stability even in the absence of additives could be highly beneficial in drug formulation and manufacturing environments.

Acknowledgements

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Chapter V – Highly concentrated lyophilized formulations

5. Protein HESylation for the use of highly concentrated and freeze-dried formulations of HES-anakinra conjugates: storage stability and the benchmark to PEGylation

All conjugation and purification steps involved in preparing the HESylated protein were performed by Sarah Bergmann, Fresenius Kabi.

Abstract

The purpose of this study was to investigate the effect of lyophilization on highly concentrated formulations of polymer-protein conjugates and their stability upon storage. PEGylation and HESylation, two strategies currently used to prolong serum half-life of proteins and peptides, were applied to the model protein anakinra. The resulting conjugates were lyophilized with or without the addition of sucrose at protein concentrations up to 50 mg/mL. Results showed that the nature of the polymer significantly impacts the colloidal stability of the conjugate. In both cases, PEGylation and HESylation prevented protein-protein interactions as assessed by lower particle counts for sizes greater than 1 μm , whereas the native protein formed significantly higher quantities of aggregates. However, in PEGylated proteins, PEG promotes conjugate crystallization during lyophilization. This behavior fostered degradation of the conjugate, resulting in the formation of high levels of soluble aggregates that could not be substantially decreased by the addition of sucrose. In contrast, HESylated anakinra remained completely amorphous and exhibited remarkable stability even over 3 months of storage at 40°C in the absence of sucrose. Additionally, coupling HES to APIs such as proteins offers a number of other advantages over PEGylation.

Keywords

HESylation, PEGylation, freeze-drying, phase separation, crystallization, aggregation

5.1. Introduction

Some high-value protein-based therapies require chronic administration of high doses of drug (several mg/kg), typically performed by intravenous or subcutaneous injection [1, 2]. The latter administration route, particularly when coupled with prefilled syringe and autoinjector devices, is restricted primarily by injection volume (≤ 1.5 mL) and therefore requires APIs to be formulated at high concentrations. Under conditions of high concentration, proteins are known to have but limited intrinsic stability, constraining the development of lyophilized formulations [1], which already experience reduced physical and chemical stability in the dried state [3-5]. For small proteins and peptides such as cytokines, antibody fragments or protein scaffolds that require half-life extension, the development of a highly concentrated and lyophilized formulation is still challenging due to a number of drawbacks stemming from PEGylation, the main HLE technology in use today. In highly concentrated aqueous solutions, PEG promotes an extraordinary increase in viscosity and can generate certain peroxides, especially at high temperatures, which accelerates degradation of the protein portion of the conjugate [6, 7]. During lyophilization, PEG tends to crystallize rapidly regardless of whether it is covalently attached or used as a bulking agent, resulting in further degradation of the protein upon storage, especially at high temperatures [6, 8, 9]. Complete suppression of crystallization requires high disaccharide-to-PEG ratios [8], which places clear limitations on the use of highly concentrated and freeze-dried formulations of PEGylated drugs. A comparative study of lyophilized PEGylated and HESylated IFN α showed the strong need for an amorphous lyoprotectant like sucrose to stabilize the PEGylated protein, whereas the HESylated form maintained an amorphous character throughout lyophilization and exhibited excellent storage stability even in the absence of additives (see chapter IV). Here, it is worth highlighting that HESylation of proteins yields an additional stabilizing effect in highly concentrated formulations of protein-polymer conjugates during freeze-drying and storage. We tested these effects using anakinra, which is usually administrated by subcutaneous injection of a highly concentrated solution (150 mg/mL) [6, 7]. For highly concentrated solutions of HESylated anakinra, we recently showed that attachment of HES significantly improves the physicochemical stability of the protein as compared to the unmodified or PEGylated versions [7]. In the present study, formulations of anakinra were subjected to an additional freeze-drying step to evaluate the stability of highly concentrated polymer-protein conjugates using the performance of PEGylated protein as a benchmark. As already described, N-terminal PEGylation and HESylation was performed by reductive amination followed by purification using anionic

exchange chromatography [7]. Additionally, both conjugates were up-concentrated, lyophilized and stored at elevated temperatures. Equivalent lyophilization and storage parameters were applied for native anakinra, which served as a control for this study.

5.2. Materials and Methods

Kineret[®] was purchased from SOBI (Stockholm, Sweden). Branched PEG propionaldehyde (MW = 40 kDa) was purchased from Jenkem Technologies (Allen/TX, USA). Activated hydroxyethyl starch (HES), with a weight average molar mass (Mw) of approximately 85 kDa, number average molar mass (Mn) of approximately 65 kDa, and a polydispersity of 1.3 [10] was from Fresenius Kabi Deutschland GmbH (Bad Homburg, Germany). All other chemicals were used in analytical grade and purchased from VWR (Germany).

5.2.1. Synthesis and purification

A detailed description of the PEGylation and HESylation processes is already provided in **chapter III** and was used without modification for the following experiments.

5.2.2. Sample preparation

Native anakinra (Kineret[®]) formulated at a protein concentration of 150 mg/mL was rebuffered into 10 mM citrate buffer, pH 6.5 using 70 mL Slide-A-Lyzer[®] dialysis cassettes (Thermo Scientific, Schwerte, Germany). Buffer exchange to 10 mM citrate, pH 6.5 of PEGylated and HESylated anakinra was performed by TFF using a 50 cm² PES (polyethersulfone) membrane capsule with a MWCO of 10 kDa operated on a Minimate[™] benchtop system (both from Pall, Dreieich, Germany) to a protein concentration of 10-15 mg/mL. The final up-concentration step for the conjugate solutions was performed by ultrafiltration in Vivaspin 20 centrifugal concentrators using a molecular weight cutoff (MWCO) of 10 kDa. The tubes were placed into a Sigma 4K15 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) and rotated at 10,000 rpm (= 14,243 g) for 3-4 h at 4°C. The protein concentration was verified by UV absorption (NanoDrop, Thermo Scientific, Schwerte, Germany) using the extinction coefficient of the protein as reported by Raibekas *et al.* [21]. For further experiments, samples were diluted in formulation buffer to the desired protein concentration. Table 1 summarizes the details of formulations used for lyophilization experiments and subsequent storage at 2-8 and 40°C for 3 months.

Sample	Protein concentration [mg/ mL]	Sucrose [% (w/v)]
Native anakinra	10	0
	10	2.5
	10	5
	25	0
	25	5
	50	0
	50	5
PEGylated anakinra	10	0
	10	2.5
	10	5
	25	0
	25	5
	50	0
	50	5
HESylated anakinra	10	0
	10	2.5
	10	5
	25	0
	25	5
	50	0
	50	5

Table 1: selected formulations for lyophilization and storage stability studies

5.2.3. Tg' measurement

The Tg' was determined by differential scanning calorimetry on a Netzsch DSC 204 Phönix (Netzsch, Selb, Germany). 20 µL of each formulation was hermetically sealed in 40 µl aluminum pans, cooled from 20 to -70°C at a cooling rate of 10°C/min and heated up to 20°C to detect the glass transition temperature of the frozen solutions.

5.2.4. Freeze-drying protocol

The freeze drying process was performed in a Martin Christ Epsilon 2-6D pilot-scale freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) using the following freeze-drying protocol. 2R vials (Vetter, Ravensburg, Germany) were filled with 0.2-0.3 mL sample volume. After shelf loading the vials were frozen by cooling at 1°C/min from 20 to -45°C and holding at the final temperature for 90 min. Primary drying

was performed at 0.1 mbar with a heating ramp from -45 to -15°C at a rate of 0.1°C/min and holding the final temperature for 30 h. Secondary drying consisted of an equivalent ramp to 20°C, followed by holding the system at 0.01 mbar for 15 h. The vials were flooded with gaseous nitrogen, stoppered with Teflon-coated stoppers (West Pharmaceutical Services, Eschweiler, Germany) at 800 mbar and afterwards manually sealed with 13 mm flip off seals (West Pharmaceutical Services, Eschweiler, Germany).

5.2.5. Tg and degree of crystallization

The glass transition temperature of the freeze-dried cakes was determined by DSC measurement. In a pressurized air flooded glove box with a relative humidity between 5-10%, a sample amount of 1-20 mg was weighed in aluminum pans and hermetically sealed. For DSC measurement the pans were heated from 10 to 110°C, cooled to the starting point and heated again to 180°C with a constant heating rate of 10°C/min. The second heating step was essential for determination of the correct glass transition temperature due to structural relaxation of the cakes (Tg overshoot). Additionally, the DSC thermograms impart information about crystallization. A 2.3% PEG solution (of the same PEG, used for conjugation) was lyophilized under equivalent conditions and analyzed by DSC measurement. The obtained enthalpy, calculated by measuring the area under the curve in J/g, was set to 100% to form the normalization basis for quantifying crystallinity in the freeze-dried PEG- protein samples.

5.2.6. Residual moisture

The moisture content of the cakes was measured by a coulometric Karl-Fischer Titrator (KF 373, Metrohm GmbH & Co, Filderstadt, Germany).

5.2.7. Turbidity measurement

Protein quality after reconstitution with highly purified water and a dilution step to 1 mg/mL in formulation buffer was evaluated using turbidity. Sample degassing was performed using a Microcal ThermoVac2 degassing station (MicroCal Inc, Northampton, MA). The turbidity of a 2 mL sample volume was determined at 860 nm and given in formazine nephelometric units (FNU) using a NEPHLA turbidimeter (Dr. Lange, Düsseldorf, Germany).

5.2.8. Particle count - Light obscuration

According to Ph. Eur. and USP specifications, particle analysis was performed by light obscuration using a PAMAS SVSS-C particle counter (PAMAS, Rutersheim, Germany) [22]. Particles with sizes of 1, 10 and 25 μm were quantified after a rinse volume of 0.5 mL in three runs of 0.3 mL with an emptying and rinse rate of 10 mL/min.

5.2.9. Monomer recovery – Size exclusion chromatography

HP-SEC was performed on a Spectra System P2000 (Thermo Scientific, Germany) equipped with a TSK guard column and a TSKgel G3000 SWxl (Tosoh Bioscience GmbH, Stuttgart, Germany). After an injection of 50 μg protein, each run was performed by an isocratic elution of the mobile phase, which contained 10 mM citrate, 140 mM NaCl, pH 6.5 at 0.5 mL/min over 45 min followed by UV detection of monomer and high molecular weight (HMW) content at 280 nm.

5.3. Results

The thermal properties of the frozen solutions were characterized by differential scanning calorimetry to detect the glass transition temperatures of the maximally freeze-concentrated matrix; this glass transition temperature is a critical process parameter, knowledge of which helps avoid cake collapse during freeze-drying. **Table 2** summarizes the obtained T_g' values of selected formulations. Covalently linked PEG chains found in PEGylated protein conjugates rapidly began to crystallize during freezing and thawing. In most cases, the prominent endothermic melting event overlapped with the predicted glass transition temperature range and therefore no T_g' could be detected. Additionally, the limited sensitivity of the instrument restricted our ability to detect rather small glass transitions of formulations containing only anakinra, lyophilized without the addition of sucrose. However, it is readily apparent that protein concentration has an impact on T_g' . For instance, raising the protein concentration of native anakinra from 10 to 50 mg/mL in the presence of 5% (w/v) sucrose increased the glass transition temperature by about 3.8 K. HESylation led to an additional elevation of the glass transition temperature; at a protein concentration of 50 mg/mL in the presence of 5% sucrose, HESylation led to elevation of the T_g' by 5.8 K.

Sample		Protein to sucrose ratio [%(w/v) / %(w/v)]	Before freeze-drying
Naitve anakinra	Tg' [°C]	1/0	*
		1/2.5	-28.1
		1/5	-28.7
		2.5/0	*
		2.5/5	-26.6
		5/0	*
		5/5	-24.9
PEGylated anakinra		1/0	*
		1/2.5	*
		1/5	-30.3
		2.5/0	*
		2.5/5	*
		5/0	*
		5/5	*
HESylated anakinra		1/0	-16.9
		1/2.5	-22.8
		1/5	-25.1
		2.5/0	-14.1
		2.5/5	-21.0
		5/0	-13.6
		5/5	-19.1

Table 2: Thermal analysis of the frozen formulations before lyophilization

5.3.1. Cake properties after lyophilization and storage

5.3.1.1. Glass transition temperature and residual moisture

Glass transition temperatures (Tg) of the lyophilized formulations, as well as the measured residual moisture (RM), are summarized in **Table 3**. In general, significantly higher RM contents can be observed for the sucrose-free formulations. Immediately after freeze-drying, native anakinra formulated with sucrose exhibited glass transition temperatures in a range between 69-84°C and residual moisture values of 0.5-1.3%, both of which remained more or

less constant during storage at 2-8°C. Upon storage at 40°C for 3 months, higher RM values were obtained. This had a plasticizing effect on the glass transition temperature, leading us to infer that water vapor from the stoppers escaped into the lyo cakes. The modifying effect induced by PEGylation and HESylation in both cases decreased the residual moisture. For both types of conjugates, higher RM values were obtained for the samples stored at 40°C, whereas lower RM numbers were found for the samples stored at 2-8°C. For PEG-anakinra, no Tg values could be measured due to the fact that a predominant melting peak of PEG overlapped the expected glass transition point. This effect could not be suppressed by the amount of sucrose used in this study. Due to the elevated Tg of HES, the glass transition temperature of HESylated anakinra exceeded 110°C for the sucrose free formulations and was substantially lowered when sucrose was added. For example, the presence of 5% sucrose at a 10 mg/mL protein concentration resulted in Tg values around 78°C. An increase in protein concentration from 10 to 25 and 50 mg/mL lowered the sucrose to polymer ratio. Lowering this ratio also reduced the plasticizing effect of sucrose on the glass transition temperature to negligible levels; all Tg values measured immediately after lyophilization at a protein concentration beyond 25 mg/mL exceeded 100°C.

Sample		Protein to sucrose ratio [%(w/v) / %(w/v)]	After freeze- drying	After 3 months storage at 2-8°C	After 3 months storage at 40°C		After freeze- drying	After 3 months storage at 2-8°C	After 3 months storage at 40°C
Native anakinra	Tg [°C]	1/0	*	*	*	RM [%]	3.30 ± 0.20	3.46 ± 0.13	7.43 ± 0.03
		1/2.5	64.7 ± 0.5	70.7 ± 7.6	55.6 ± 15.8		1.27 ± 0.33	1.12 ± 0.06	4.05 ± 0.48
		1/5	64.7 ± 0.2	72.7 ± 0.3	46.5 ± 5.9		0.83 ± 0.10	0.91 ± 0.03	2.24 ± 0.07
		2.5/0	*	*	*		2.55 ± 0.38	2.77 ± 0.79	5.43 ± 0.41
		2.5/5	84.1 ± 0.1	64.7 ± 9.6	51.7 ± 2.0		0.90 ± 0.23	0.97 ± 0.11	2.69 ± 0.40
		5/0	*	*	*		2.26 ± 0.21	1.96 ± 0.45	4.36 ± 0.24
		5/5	80.0 ± 22.7	79.5 ± 0.7	61.0 ± 3.6		0.47 ± 0.19	1.13 ± 0.02	2.65 ± 0.27
PEGylated anakinra		1/0	*	*	*		1.39 ± 0.49	0.98 ± 0.06	2.25 ± 0.27
		1/2.5	*	*	*		0.80 ± 0.01	0.64 ± 0.08	2.27 ± 0.34
		1/5	*	*	*		0.76 ± 0.30	0.72 ± 0.02	2.23 ± 0.05
		2.5/0	*	*	*		0.81 ± 0.11	0.71 ± 0.07	1.71 ± 0.16
		2.5/5	*	*	*		0.57 ± 0.02	0.73 ± 0.03	1.88 ± 0.04
		5/0	*	*	*		0.97 ± 0.71	0.59 ± 0.20	1.33 ± 0.05
		5/5	*	*	*		0.42 ± 0.05	0.46 ± 0.07	1.43 ± 0.21
HESylated anakinra		1/0	110.3 ± 1.9	109.2 ± 0.2	*		0.91 ± 0.04	0.92 ± 0.04	3.38 ± 0.43
		1/2.5	94.1 ± 0.9	86.2 ± 2.3	61.5 ± 10.0		0.63 ± 0.23	0.68 ± 0.01	2.86 ± 0.01
		1/5	77.8 ± 5.2	78.4 ± 0.1	49.8 ± 2.3		0.45 ± 0.01	0.53 ± 0.06	2.37 ± 0.21
		2.5/0	110.8 ± 0.6	111.6 ± 2.9	113.8 ± 0.6		0.35 ± 0.04	0.86 ± 0.04	2.75 ± 0.27
		2.5/5	100.9 ± 7.2	89.5 ± 1.8	62.8 ± 1.3		0.29 ± 0.01	0.60 ± 0.11	2.24 ± 0.04
		5/0	110.7 ± 1.3	110.4 ± 2.2	112.1 ± 0.6		0.60 ± 0.15	0.57 ± 0.24	2.02 ± 0.25
		5/5	109.9 ± 10.0	110.5 ± 4.0	66.5 ± 0.6		0.41 ± 0.02	0.39 ± 0.05	2.04 ± 0.23

* not detectable

Table 3: Glass transition temperatures and residual moisture immediately after lyophilization and after 3 months storage at 2-8 and 40°C

5.3.1.2. Degree of crystallization

During freeze-drying of native and HESylated anakinra, no crystallization could be detected by DSC analysis. In contrast, PEGylated anakinra rapidly began to crystallize during lyophilization, an effect which could only be suppressed by the addition of sucrose to a certain polymer-to-disaccharide ratio (Table 4). Increasing the amount of PEG in the formulation substantially lowered the sucrose-to-PEG ratio and therefore enhanced the role of crystallization in the lyo cake. Samples, which were stored at 40°C showed, as expected, a slight increase in crystallization. Interestingly, during storage at 2-8°C the degree of crystallization was in some cases lower than the initial value obtained immediately after freeze-drying.

Sample		Protein to sucrose ratio [%(w/v) / %(w/v)]	After freeze- drying	After 3 months storage at 2-8°C	After 3 months storage at 40°C
Native anakinra	Crystallinity [%]	1/0	*	*	*
		1/2.5	*	*	*
		1/5	*	*	*
		2.5/0	*	*	*
		2.5/5	*	*	*
		5/0	*	*	*
		5.0/5	*	*	*
PEGylated anakinra		1/0	64.4 ± 1.7	67.4 ± 0.3	68.1 ± 0.9
		1/2.5	38.7 ± 0.1	39.0 ± 0.3	43.7 ± 2.0
		1/5	23.2 ± 1.7	16.3 ± 1.9	29.1 ± 0.3
		2.5/0	66.0 ± 2.3	68.9 ± 0.8	68.3 ± 1.6
		2.5/5	44.4 ± 0.2	44.7 ± 0.5	44.5 ± 1.9
		5/0	66.7 ± 0.3	67.7 ± 3.8	71.1 ± 1.5
		5.0/5	57.2 ± 0.2	53.0 ± 1.2	58.4 ± 0.2
HESylated anakinra		1/0	*	*	*
		1/2.5	*	*	*
		1/5	*	*	*
		2.5/0	*	*	*
		2.5/5	*	*	*
		5/0	*	*	*
		5.0/5	*	*	*

* not detectable

Table 4: Thermal analysis for the degree of crystallization

5.3.2. Protein quality after reconstitution

5.3.2.1. Colloidal stability after lyophilization and storage

The number of particles with a size greater than 1 μm and corresponding turbidity values were measured immediately after lyophilization and subsequent storage (**Figure 1**). Especially for native anakinra, storage temperature and the presence of sucrose had the biggest impact on the degree of aggregate formation. Formulations lacking sucrose exhibited greater numbers of particles, particularly at protein concentrations of 25 and 50 mg/mL. Obtained particle counts are in good agreement with the turbidity measurements; higher particle numbers are associated with higher turbidity values. These effects are more distinct for samples which were stored at 40°C. The addition of sucrose substantially decreased both particle counts and the solution turbidity, indicating the strong need for a lyoprotectant in the formulation. In contrast, conjugation with either PEG or HES prevented the formation of aggregates. This effect can be explained by the high shielding effect of both polymers. As one might expect, then, correspondingly low particle counts were observed in each conjugate formulation.

5.3.2.2. Monomer recovery and soluble aggregates

Monomer recovery and levels of soluble aggregates were monitored by HP-SEC analysis. Protein concentration and the amount of sucrose were found to have the greatest influence on the formation of subvisible particles (**Figure 2**). While native anakinra showed no substantial loss in monomer content, an increase in soluble aggregates was observed especially for formulations, lyophilized in the absence of sucrose. Including sucrose in the formulation substantially lowered the degree of aggregate formation. In contrast, PEGylated anakinra exhibited both dramatic loss in monomer recovery and rapid formation of soluble aggregates especially for samples lyophilized without sucrose (the reader is advised to note the difference in y-axis scales in the presented figures). Again, sucrose is found to prevent formation of particles and preserve high monomer levels. Interestingly, the loss in monomer recovery correlates well to the degree of crystallization measured via DSC. When crystallization of PEG is not suppressed by amorphous excipients, the PEGylated protein degrades rapidly during long-term storage. Surprisingly, even in the absence of sucrose, HESylation of anakinra neither diminished monomer recovery nor promoted formation of high levels of soluble aggregates.

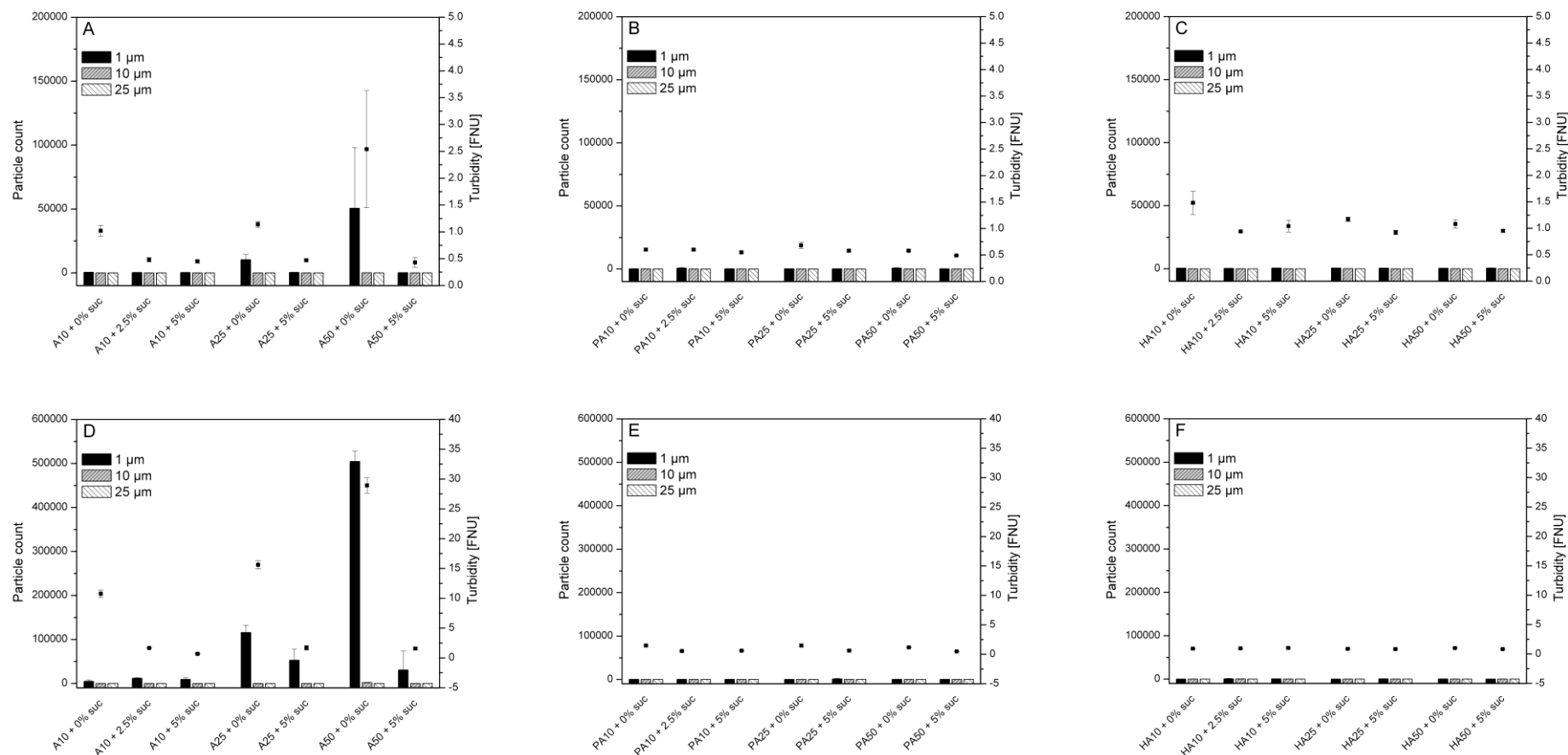


Figure 1: Turbidity and particle counts immediately after lyophilization (A) native anakinra; (B) PEGylated anakinra and (C) HESylated anakinra; Turbidity and particle counts after storage at 40°C for 3 months (D) native anakinra; (E) PEGylated anakinra and (F) HESylated anakinra; X axes keys: A: native anakinra; PA: PEG- anakinra; HA: HES-anakinra and 10, 25 or 50 represent the protein concentration in mg/mL

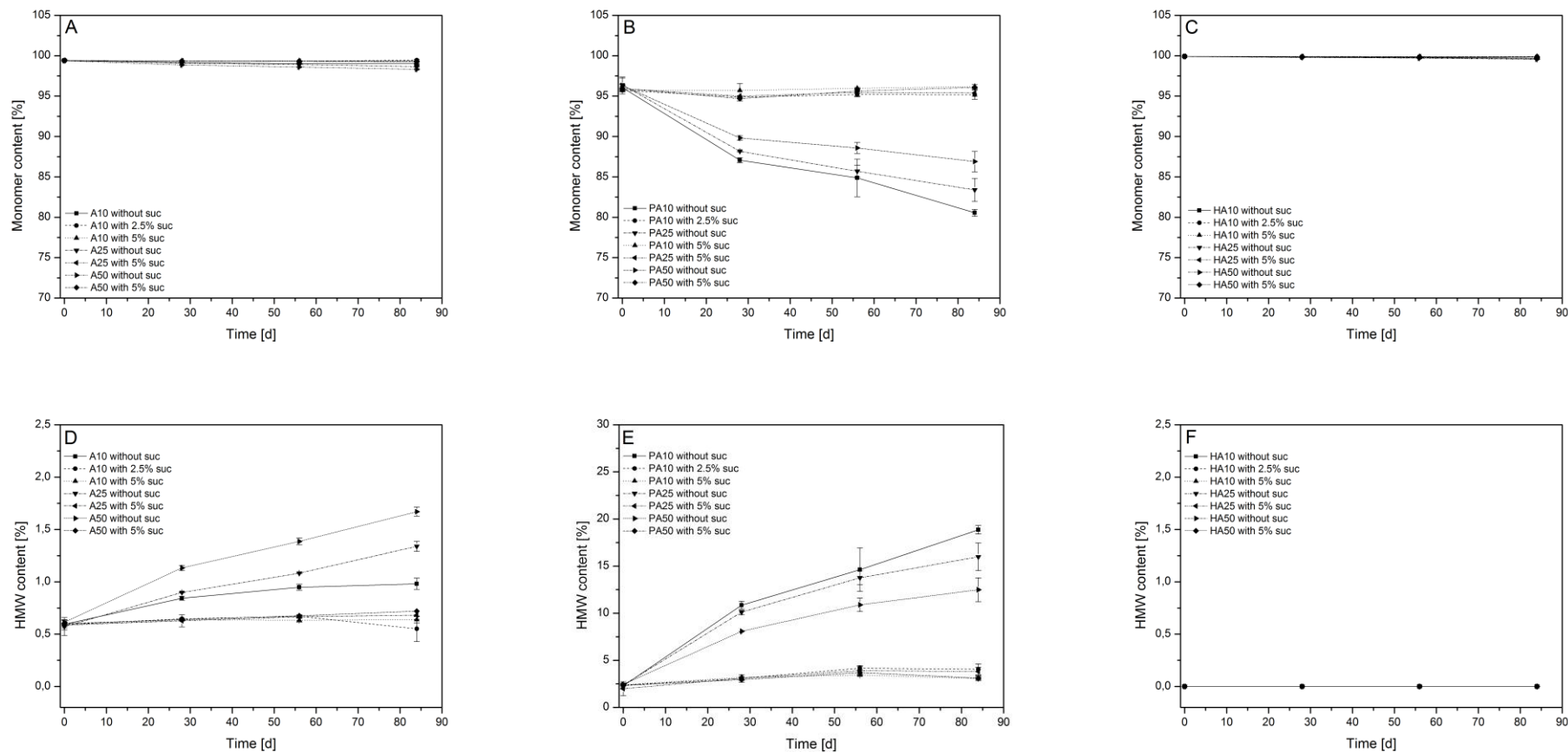


Figure 2: SEC analyses after 3 months at 40°C: monomer content of (A) native anakinra (B) PEGylated anakinra and (C) HESylated anakinra; high molecular weight (HMW) content in (D) native anakinra (E) PEGylated anakinra and (F) HESylated anakinra; Caption key: A: native anakinra; PA: PEG-anakinra; HA: HES-anakinra and 10, 25 or 50 represent the protein concentration in mg/mL

5.4. Discussion

The development of PEGylated drugs in highly concentrated formulations represents an ongoing challenge due to the high viscosity of PEG at high concentrations, hampering manufacturing processes and drug injectability [6]. For the use of freeze-dried formulations of PEGylated proteins, high disaccharide-to-PEG ratios are necessary to obtain a reasonable shelf-life due to fact that PEG will rapidly crystallize during freeze-drying, which may decrease storage stability and induce protein degradation if left unsuppressed by amorphous lyoprotectants [8].

The main focus of this study was to apply HESylation technology to the formulation of highly concentrated, freeze-dried protein-polymer conjugates. Freeze-drying is a time- and cost-intensive process, and the glass transition temperature of the maximally freeze-concentrated phase has a considerable effect on the overall drying time. To reduce drying time, the target product temperature during primary drying should be as high as possible while staying well below the glass transition temperature to yield a pharmaceutically elegant cake with no obvious meltbacks or sample collapse [11]. A nearly 1°C increase in product temperature lowers the time for primary drying by about 13% [4]. As described previously, an increase in protein concentration will increase the T_g' and therefore a higher T_p can be applied for primary drying [12, 13]. We confirm these findings with our results for the case of native anakinra. An additional enhancement is observed upon protein HESylation. In general, HES solutions are characterized by high T_g' values in a range of -12 to -17°C depending on the molecular weight, the degree of substitution and the HES-to-disaccharide ratio [14-17]. For highly concentrated formulations, the elevating effect of HESylation on T_g' was highly significant, thus enabling higher T_p values during the lyophilization process and a concomitant reduction in the time required for primary drying. This cannot be presently achieved using either the native or PEGylated protein. To ensure appropriate storage stability in a dried state, the glass transition temperature should be as high as possible. Typically, the glass transition should be at least 20°C above ambient temperature to limit molecular mobility in the freeze-dried cake and thus prevent protein degradation [15, 18, 19]. The glass transition temperatures we obtained immediately after lyophilization satisfy this criterion and match previously reported glass transition points of sucrose-based formulations [20]. In addition, an increase in protein concentration has been reported to generally enhance the glass transition temperature [21]. We confirm this finding for anakinra; higher protein concentrations showed higher T_g values. The effect of HESylation, as expected, further increased the glass transition temperature, an effect

which has already been shown for HESylated IFN α -2b (see **chapter IV**). HES itself has a very high Tg, which depends on molecular weight and degree of substitution. This modifying effect on glass transition temperature is directly transferred to covalently modified HES-protein conjugates,. Thus, the presence of HES opposes the plasticizing effect of water in the lyo cake and maintains the glass transition point above the storage temperature. This effect has the potential to make an impact on next-generation drying methods, where in most cases higher residual moisture contents can be expected after the drying process [22]. Methods such as spray-, convective, vacuum and microwave drying or combinations thereof are mainly focused on overcoming the drawbacks of lyophilization, for example, by reducing the overall process time or the high purchase and maintenance costs of the drying equipment. Additionally, freeze-drying has a low energy efficiency and can cause a significant loss in API quality due to its exposure to various process-related stresses such as the freezing step [22].

One potential limitation of polyethylene glycol is its strong tendency to crystallize during lyophilization and subsequent storage, fostering degradation of the conjugated protein [8, 9, 23]. This effect can be overcome by the use of amorphous lyoprotectants such as sucrose in disaccharide-to-PEG weight ratios ≥ 5 [8]. Protein degradation occurring as a result of crystallization is mainly induced by interfacial denaturation at the large ice/water interface [24, 25]. In contrast to PEGylation strategies, crystallization cannot be observed when HES is attached to the protein. In a similar fashion to dextran, HES is based on a carbohydrate scaffold and therefore acts as a completely amorphous bulking agent during freeze-drying [13, 26]. The effect of covalent modification on colloidal stability of conjugate formulations was evaluated by turbidity measurement, particle counting and HP-SEC for monomer recovery [27, 28]. PEGylation and HESylation of proteins tend to prevent protein-protein interaction and reduce protein aggregation in aqueous solution [7, 10, 29-31]. In the current study, both modifications of anakinra exhibited this effect. PEGylated and HESylated anakinra both generated significantly lower particles counts upon lyophilization and subsequent storage than the unmodified protein. HP-SEC provides information on entities existing at the submicron level, including whether or not the attachment of PEG or HES highly influences the formation of aggregates. For PEG-containing samples, which tend to experience heavy crystallization and concomitant protein degradation, significant losses in monomer recovery were observed. In contrast, HES-containing conjugates did not show any tendency toward crystallization and thus inhibited the formation of both submicron and micron-scale aggregates. Thus, for all chosen protein concentrations, even in the absence of sucrose, no significant loss in monomer recovery could be observed. These results make feasible the use of formulations with high

protein loads, for applications such as further integration of lyophilisates into drug delivery systems. In addition, the absence of a requirement for lyo excipients enables the use of freeze-drying for up-concentration to obtain highly concentrated formulations of HES-protein conjugates.

5.5. Conclusion

Lyophilization makes proteins susceptible to degradation because both physical and chemical stability are significantly reduced in the dried state. The use of chemical modifications such as polymer conjugation can prevent protein aggregation in the liquid state by hampering PPIs. It has been shown that polymers such as PEG and HES differ widely in a variety of relevant physicochemical properties – variations which can either prevent or foster protein degradation after lyophilization. We showed here that in the context of highly concentrated lyophilisates, covalent linkage of HES to the model protein anakinra has several advantages. The presence of HES increases the T_g' of the solutes, permitting primary drying to be run at higher product temperatures and thus, in shorter periods of time. HES also maintains its amorphous nature after drying; cakes of HES conjugates show very high glass transition temperatures, which helps provide sufficient storage stability even in the presence of higher moisture contents. HESylation in this case enabled freeze-dried formulations without sucrose, opening the door to routine up-concentration of protein solutions. This feature is attractive for its impact on subsequent manufacturing steps, such as bulk drying of drug substance or preparation of drug delivery systems such as implants or microspheres. Beyond its excellent drying behavior, the attachment of HES prevents PPIs after reconstitution due to effective shielding. In conclusion, this study reveals that HESylation has the potential to replace PEGylation, especially for applications where highly concentrated lyophilisates of polymer-protein conjugates are desirable or essential.

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Chapter VI – Summary and attachments

6. Summary of the thesis

During the last decades, grafting of biocompatible polymers like polyethylene glycol to therapeutic proteins and peptides for half-life extension has become an established and highly refined technology. With over 10 approved PEGylated drugs, this technology is considered to be the gold standard for half-life extension. However, PEG has a number of serious shortcomings, which have recently spurred development of alternative HLE technologies based on both biocompatible and biodegradable polymers. Such alternatives aim to prolong the circulation time of small biologics while simultaneously overcoming formulation challenges posed by use of PEG. In response, the overall goal of this thesis is to further develop HESylation technology and overcome some of the challenges associated with formulation of novel, biodegradable HES-protein conjugates. Results from preliminary studies revealed that the chemical attachment of HES is a promising tool for serum half-life modulation of proteins and offers distinct formulation advantages over PEGylation.

In the first phase of the project hydroxyethyl starch (HES) was chemically grafted to a model protein called anakinra. Chapter II describes the synthesis and physicochemical characterization of the HESylated protein. Site-specific HESylation of anakinra at the N-terminus was achieved using reductive amination at a pH of 5.0. The mildly acidic conditions during synthesis and subsequent purification by anionic exchange chromatography at a pH of 8.0 had no significant influence on either the protein conformation or its biological affinity to its cognate receptor. Coupling HES to anakinra improved the thermodynamic properties, prevented protein aggregation during accelerated stress conditions and prolonged the pharmacokinetics of the protein *in vivo*.

In a next step, HESylated anakinra was benchmarked against its PEGylated counterpart with respect to the effect on the conjugate's physicochemical properties in highly concentrated solutions, where protein stability and solution viscosity can be notably suboptimal. HESylated and PEGylated anakinra were synthesized and purified under equivalent conditions. Coupling a 40 kDa branched PEG and an 85 kDa HES to anakinra resulted in conjugates of comparable hydrodynamic size. Highly concentrated formulations of both conjugates exhibited increased melting temperature in solution, which led to improved thermodynamic stability when compared to the unmodified counterpart. However, solution viscosity and colloidal stability completely differed for both conjugates. In general, more highly concentrated protein solutions are associated with a greater tendency toward aggregation as a result of molecular crowding. In addition, reversible self-association in more crowded conditions makes solutions of such pro-

teins liable to show an increase in viscosity. The presence of a covalently coupled polymer aggravates changes in viscosity due to entanglement of adjacent polymer chains. This phenomenon is more pronounced for the highly flexible polymer chains found in PEG molecules. In contrast, the highly branched polymeric architecture of HES provides a stiffer structure in which the molecules act more like a hard sphere. As a result, the viscosity of HESylated protein solutions was observed to be much lower than that of solutions of PEG conjugates at equivalent protein concentration. Although both conjugates showed improved monomer recovery, HESylation provided for higher colloidal stability compared to native and PEGylated anakinra at all studied concentrations. The root cause for this surprising difference in protein stability might be the difference in the nature of the two polymers. PEG is known to change the polarity and dielectric constant of aqueous solutions and can generate peroxides, especially at high temperatures, which could have led to the higher degree of protein degradation observed in comparison to the HESylated protein. The combination of lower intrinsic viscosities and improved storage stability of the HES conjugate reveals that HESylation can provide formulation advantages over PEGylation, especially for the development of highly concentrated formulations of polymer-protein conjugates.

The final part of the project aimed to distinguish between the physicochemical properties of lyophilized PEGylated and HESylated proteins. In this study the model protein interferon α -2b was either PEGylated or HESylated in a site-specific manner, lyophilized and stored under elevated temperatures (2-8 and 40°C) for three months. In chapter IV we studied the effect of lyoprotectants by varying the amount of sucrose in the freeze-dried formulations. We also examined the influence of storage temperature and evaluated how conjugation of each polymer impacts the conformational and colloidal stability of the protein. Our results show that PEG tends to crystallize upon freeze-drying, which has a significant influence on protein stability during storage, especially at high temperatures. The amount of crystallization could be substantially limited by formulating with sucrose at high disaccharide-to-PEG ratios. Consequently, samples of the PEGylated protein lyophilized without the addition of sucrose and stored at 40°C showed the highest degradation rate. In conclusion, freeze-dried formulations of PEGylated proteins require large amounts of amorphous lyoprotectants to enable sufficient storage stability. In contrast, HESylation of IFN α drastically improved the colloidal stability of the protein by reducing monomer loss and tendency toward aggregation. This can be explained by two facts: first, HES remains totally amorphous during lyophilization and second, it provides a protective shield that prevents PPI when covalently attached to the protein. Additionally, HES exhibits very high initial glass transition temperatures both in solution and in

the dried state – properties which may be directly transferred to the conjugate. An increase in the T_g' of frozen solutions makes a more economic lyophilization cycle possible by enabling application of higher product temperatures, which reduces the time required for primary drying. The presence of HES in the conjugate also provides a protective shielding that reduces aggregate formation, even in the absence of sucrose and under storage at high temperatures. Our results show that it might even be possible to formulate HESylated interferon α -2b without any sugar at all. A sucrose-free, freeze-dried formulation would permit a very high protein load, which is extremely attractive for further manufacturing steps; examples include integration with drug delivery systems such as implants. In conclusion, the results of this study reveal that HESylation can provide formulation advantages over PEGylation during lyophilization.

In chapter V we investigated the stability of highly concentrated PEGylated and HESylated anakinra upon freeze-drying. After storage at 2-8 and 40°C, both polymers imparted effective shielding and suppression of micron-scale aggregate formation. However, due to the high tendency of PEG to crystallize during lyophilization, an effect which could not be fully suppressed by the use of certain amounts of sucrose, the PEGylated protein degraded rapidly and formed large amounts of soluble submicron aggregates. In the presence of sucrose, native anakinra showed a reduced tendency toward aggregation, as seen via lower particle counts. Formulations of the HESylated protein remained remarkably stable and did not promote the formation of aggregates on either micron or submicron scales, even in the absence of sucrose. Lyophilization studies of both dilute and highly concentrated HESylated protein solutions confirmed the benefit of covalently bound HES. Taken together, these results paint an extremely attractive portrait of the potential benefits of incorporating HESylation technology into biologic formulation development workflows, and should motivate further studies in the field of HESylated drugs.

7. Publications associated with this thesis

7.1. Research articles

Robert Liebner, Roman Mathaes, Martin Meyer, Thomas Hey, Gerhard Winter, Ahmed Besheer Protein HESylation for half-life extension: Synthesis, characterization and pharmacokinetics of HESylated anakinra – *European Journal of Pharmaceutics and Biopharmaceutics*. 2014, 87(2): 378-385

Robert Liebner, Martin Meyer, Thomas Hey, Gerhard Winter, Ahmed Besheer Head to head comparison of the formulation and stability of concentrated solutions of HESylated versus PEGylated Anakinra – *Journal of Pharmaceutical Sciences*. 104 (2015) 515-526

Robert Liebner, Sarah Bergmann, Thomas Hey, Gerhard Winter, Ahmed Besheer Freeze-drying of HESylated IFN α -2b: Effect of HESylation on storage stability and the benchmark to PEGylation – *In preparation*

7.2. Book chapter

Ahmed Besheer, Robert Liebner, Martin Meyer, Gerhard Winter Challenges for PEGylated Proteins and Alternative Half-Life Extension Technologies Based on Biodegradable Polymers - *Tailored Polymer Architectures for Pharmaceutical and Biomedical Applications*. 2013, 1135(13): 215-233

8. Presentations associated with this thesis

8.1. Oral presentations

Robert Liebner, Gerhard Winter, Ahmed Besheer
Head to head comparison of HESylated and PEGylated Proteins
Biacore™ and MicroCal™ User meeting 2013, 4th and 5th of June 2013, Weimar, Germany

Robert Liebner, Gerhard Winter, Ahmed Besheer

Head to head comparison of HESylated and PEGylated proteins

DPhG annual conference 2013, 9th and 11th of October 2013, Freiburg im Breisgau, Germany

8.2. Poster presentations

Robert Liebner, Gerhard Winter, Ahmed Besheer

Synthesis and physicochemical characterization of PEGylated anakinra - 8th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, 19th to 22nd of March 2012, Istanbul, Turkey

Robert Liebner, Elisabeth Härtl, Gerhard Winter, Ahmed Besheer

Characterization of highly concentrated therapeutic protein solutions – Bayern Innovativ/ Biopharmaceutics: Strategien – Technologien – Märkte, 23th of May 2012, Benediktbeuern, Germany

Ahmed Besheer, Robert Liebner, Gerhard Winter

Synthesis, characterization and formulation of highly concentrated solutions of PEGylated anakinra – 9th International Symposium on Polymer Therapeutics, 25th - 28th of May 2012, Valencia, Italy

Robert Liebner, Roman Mathaes, Gerhard Winter, Ahmed Besheer

Head to head comparison of PEG- and HESylated anakinra – 2013 Colorado Protein Stability Conference, 8th – 11th of July 2013, Breckenridge, CO, USA

Robert Liebner, Gerhard Winter, Ahmed Besheer

HESylation[®] of biologics: A promising alternative to PEGylation – 9th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, 31st – 3rd of April 2014, Lisbon, Portugal